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ABSTRACT: Knowledge of the ability of the female reproductive system to metabolize polycyclic aromatic hydrocarbons (PAHs) is critical to the diagnosis and management of female infertility and for risk assessment purposes. The PAHs are a family of widespread pollutants that are released into the environment from automobile exhausts, cigarette smoke, burning of refuse, industrial emissions, and hazardous waste sites. In exposed animals, PAHs become activated to reactive metabolites that interfere with target organ function and as a consequence cause toxicity. The extent of susceptibility to PAH exposure may depend on the ability of animals to metabolize these chemicals. The present study has been undertaken to assess whether any differences exist among mammals in the metabolism of benzo(a)pyrene (BaP), a prototypical PAH compound. Microsomes isolated from the liver and ovaries of rats, mice, goats, sheep, pigs, and cows were incubated with 5 μM BaP. Postincubation, samples were extracted with ethyl acetate and analyzed for BaP/metabolites by reverse-phase HPLC with fluorescence detection. The rate of metabolism (pmol of metabolite/min/mg protein) was found to be more in liver than in ovary in all the species studied ($P < 0.05$). The differences in metabolite concentrations were statistically significant ($P < 0.0001$) among the various species in both organs studied. Multiple species comparison also revealed that the differences were statistically significant ($P < 0.001$) between rodents (rat and mouse) and higher mammals (ewe, sow, and cow). Even among the higher mammals, in a majority of the cases, the differences in metabolite concentrations were significantly different ($P < 0.001$) both in ovary and liver. The BaP metabolites identified were 4,5-diol; 7,8-diol; 9,10-diol; 3-hydroxy BaP; and 9-hydroxy BaP. The rodent microsomes produced considerably higher proportion of BaP 4,5-diol and 9,10-diol than did cow, sow, goat, and sheep. However, microsomes from higher mammals converted a greater proportion of BaP to 3-hydroxy and 9-hydroxy BaP, the detoxification products of BaP. Overall, our results revealed a great variation among species to metabolize BaP. Environ Toxicol 24: 603–609, 2009.

Keywords: benzo(a)pyrene; polycyclic aromatic hydrocarbons; ovarian microsomes; liver microsomes; metabolism; HPLC

INTRODUCTION

In recent years, environmental toxicants have been implicated in various reproductive disorders in humans (Younglai et al., 2007). The difficulties encountered in studying the mechanism of action of environmental chemicals on human reproduction necessitate employing

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laboratory animals and animal models for mechanistic reproductive studies (WHO, 2001). Therefore, understanding the mechanism by which environmental toxicants are metabolized in the female reproductive system of animals will be of importance in the diagnosis and management of female fertility issues. Additionally, in reference to environmental health issues, chemical-specific metabolism data will aid in the generation of a compendium of interspecies default factors used for risk assessment (Walton et al., 2001).

Some of the environmental chemicals act as reproductive toxicants in that they bind to the receptors necessary for the regulation of reproductive organs, thus contributing to impairment in sexual behavior, gamete function, and delivery of the new born. One such environmental chemical that has been categorized as a reproductive toxicant is benzo(a)pyrene (BaP; Charles et al., 2000; Archibong et al., 2002; Inyang et al., 2003; Ramesh et al., 2008). Automobile exhausts, cigarette smoke, biomass burning, charcoal-broiled meat, industrial emissions, municipal incinerators, and hazardous waste sites contribute considerable amounts of this chemical (IPCS, 1998) to human exposure. In exposed animals and humans, BaP becomes activated in organs particularly the ovaries, to reactive metabolites that interfere with the latter organ function (reviewed in Ramesh et al., 2004) and as a consequence cause ovarian failure.

The rationale for conducting this study was to assess the qualitative and quantitative differences among different mammalian species in ovarian and hepatic microsomal metabolism of BaP, a prototypical polycyclic aromatic hydrocarbon (PAH) compound. Association has been established between the substantially higher levels of PAH exposures with increased incidence of female reproductive toxicity in laboratory animals (Borman et al., 2000; Jurisicova et al., 2007; Tsai-Turton et al., 2007) and humans (Matikainen et al., 2001; Neal et al., 2007). Therefore, information on the ability of these mammals to metabolize BaP will be useful for assessing risks to humans arising from different sources of exposure to this PAH (occupational, environmental exposures, and consumption of contaminated meats).

MATERIALS AND METHODS

Test Species/Tissue Samples

Microsomes were isolated from the liver and ovarian tissues of the following animals: rat (age = 10 weeks), mouse (age = 10 weeks), sheep (age = 20–30 months), goat (age = 16–24 months), pig (age = 9 months), and cow (age = 36 months).

Pig ovary and liver samples were obtained from the USDA Meat Animal Research Center, Clay Center, NE; cow, sheep, and goat ovary and liver samples were obtained from abattoirs in and around Nashville, TN. Similar organ samples from rat and mouse were obtained from animals maintained in-house. All animals used in this study were mature and healthy and were not subjected to any form of treatment/medication. Except for tissue samples collected from the abattoirs, the rest of the tissues used in this study were collected from animals whose uses for experiments were approved by the respective Institutional Animal Care and Use Committees.

Preparation of Microsomes

Ovary and liver samples from autopsied animals (rat and mouse), and animals from the USDA facility and abattoirs after sacrifice and evisceration (cow, sheep, goat, and pig) were immediately frozen on dry ice and stored at −70°C until isolation of microsomes. Microsomal fraction preparation and analysis were conducted simultaneously on tissues from representative species mentioned above, in order to minimize procedural variations. A minimum of five samples/organ/species were used for analysis.

Microsomal fractions were prepared, and processed as described by Schenkman and Jansson (1999) with some modifications. The connective tissues surrounding the ovary and fat on liver samples were removed following which, they were washed in chilled (4°C) isotonic saline to remove excess blood. Each ovary and liver samples were individually cut into small pieces using sterile scalpel blades, minced separately with a fine pair of scissors and thoroughly mixed to obtain a homogenous mixture of minced tissue samples per animal. One gram of each minced sample was chilled in isotonic saline for 5 min prior to being homogenized in two volumes of sucrose-TKM buffer (sucrose 0.25 M, Tris 80 mM, KCl 25 mM, $MgCl_2$ 5 mM, pH 7.4). Each minced tissue sample homogenate was centrifuged at 10 000 × *g* for 10 min, supernatant harvested and subjected to a second centrifugation at 15 000 × *g* for 15 min to pellet down nuclei and mitochondria. Each resultant supernatant was centrifuged at 100 000 × *g* for 60 min at 4°C following which, the cytosolic supernatant and microsomal pellet were separated. Each microsomal pellet was rinsed twice with 5 mL of sucrose-TKM buffer and resuspended in 5 mL of the same buffer. Ovarian or liver microsomes were aliquoted into cryovials (Wheaton Science Products, Millville, NJ), and stored frozen at −80°C until used for studying BaP metabolism. Protein content of each microsomal preparation was determined according to the method of Bradford (1976).

Microsomal Incubations and Metabolism Studies

Pilot studies were conducted to establish optimal conditions for microsomal protein concentration, substrate

concentration, and time of incubation of reaction mixtures in the assay. The results (data not shown here) showed that reaction rates were consistent with linearity of metabolism occurring in the first 15 min, at microsomal protein and substrate concentrations of 0.5 mg/mL and 5 μM, respectively.

Before conducting metabolism studies, microsomes were thawed at room temperature and 120 μL of the microsomal pellet resuspended in TKM buffer (final protein concentration = 0.5 mg/mL) were added to 5 mL of cocktail containing NADPH (0.72 mM), EDTA (100 mM), KPO4 (100 mM), $MgCl_2$. $6H_2O$ (3.75 mM). Samples per animal were preincubated for 2 min at 37°C in a tissue shaker (Precision Scientific Instruments, Chicago, IL) prior to being distributed to a treatment and a control reaction tubes in a 2 × 3 × 7 factorial arrangement (two organs, three classes of treatment, and seven species). Treatment consisted of exposure *in vitro* to 5 μM BaP (CAS No. 50-32-8; 98% pure, Sigma Chemical Co., St. Louis, MO) dissolved in dimethyl sulfoxide. After 15 min incubation at 37°C, the reaction was stopped with 8 mL of ethyl acetate containing butylated hydroxytoluene (0.2 mg/mL). Benzo(a)pyrene metabolites were extracted twice with ethyl acetate. The organic layer was removed and evaporated under nitrogen. Each sample residue was dissolved in 500 μL methanol, passed through Acrodisc filters (0.45 μm; 25 mm diameter, Gelman Sciences, Ann Arbor, MI), and subjected to reverse-phase HPLC for analyses of BaP metabolites as described previously (Ramesh et al., 2001). Benzo(a)pyrene metabolite standards were obtained from the National Cancer Institute Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). Though this analytical method was set-up originally for the rat, it was optimized for other species analyzed in our laboratory (Walker et al., 2006). Controls were processed in parallel with experimental samples under identical assay conditions. Dimethyl sulfoxide (vehicle for BaP) was used as a negative control while reaction mixtures without BaP or microsomes served as positive controls. All incubations were performed in triplicate for each ovarian and hepatic microsomal sample. The variability among triplicates was less than 10%. The rates of BaP metabolism (formation of BaP metabolites) were expressed as pmol of product formed per min per mg of microsomal protein. Because BaP and its metabolites are suspected carcinogens, they were handled in accordance with NIH guidelines for preventing exposure of laboratory personnel to this chemical (NIH, 1981).

Effect of Estrous Cycle on BaP Metabolism

To delineate the influence of different phases of estrous cycle on BaP metabolism, ovaries from commercial crossbred prepubertal gilts (7–8 month old; ovaries with follicles and no corpora lutea) and those of postpubertal gilts (ovaries with corpora lutea of various stages of development or corpora albicantia) were used.

Statistics

Data on microsomal mean BaP metabolite concentrations were analyzed by ANOVA with repeated measures and the differences among means were tested for significance by orthogonal contrasts (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

The concentration of BaP (5 μM) used in the present study is higher than the actual concentrations (0.025–0.086 ng/g wet tissue) reported in the liver, lung, and breast of healthy humans and smokers (Beach et al., 2000; Goldman et al., 2001). Nonetheless, this concentration was chosen because of its relevance to environmental levels of BaP in hazardous waste sites, former industrial sites, and exposure concentrations for ''at risk'' populations such as smokers and occupationally exposed individuals (IPCS, 1998; Ramesh et al., 2004).

Before incubation of microsomes with BaP, ovarian tissue homogenates were analyzed for the presence of BaP parent compound or its metabolites to ensure their absence in as much as they (BaP and its metabolites) could be imposed by diet on organs of farm animals in particular. Subsequent to incubation of microsomes with BaP, no unmetabolized BaP (parent compound) was detected. Regardless of species, ovary and liver microsomes exhibited the capability to metabolize BaP based on detected levels of metabolites, which were absent in the control reaction mixtures ($P < 0.005$). The concentrations of BaP metabolites produced by the ovarian microsomes varied among the species. The *in vitro* metabolism of BaP was highest in the cow and lowest in the mouse (cow > pig > goat > sheep > rat > mouse; treatment × species interaction, $P < 0.005$). Compared to rodents, the BaP metabolite concentrations produced by porcine, ovine, and bovine ovarian microsomes were higher ($P < 0.05$; Fig. 1). The total BaP metabolite concentration profiles of hepatic microsomes for the above mentioned species were similar to those of their respective ovaries. The overall metabolite concentrations in liver were species-dependent and rodents had the least concentration of BaP metabolites ($P < 0.05$; treatment × organ × species) compared with the other species. Substantial differences in ovarian and liver microsomal metabolism of BaP exist among the various species tested. Results of cross-species comparison of metabolite concentrations are shown in Figure 2. Data reported in Figure 1 was subjected to individual species-wise comparison, which showed that the differences in metabolite concentrations were statistically significant ($P < 0.0001$) in the two organs studied.

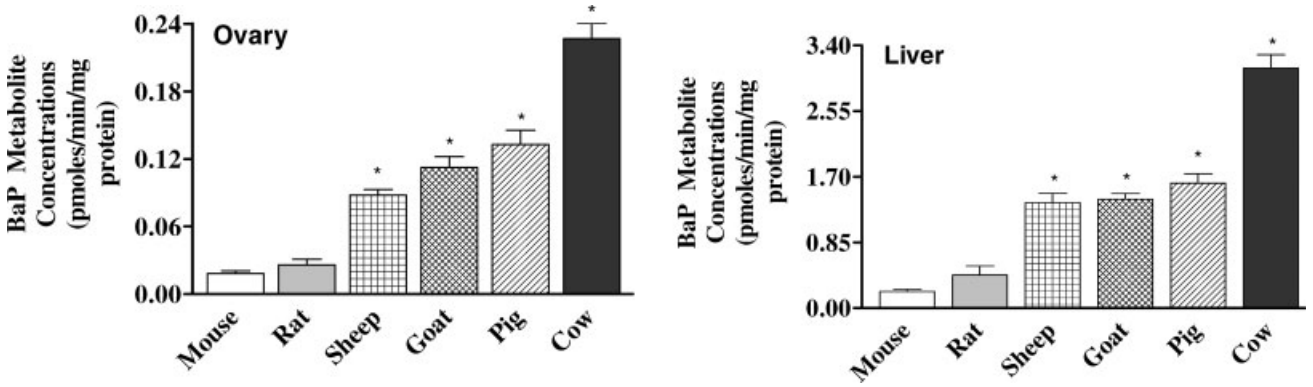

Fig. 1. Comparison of the metabolism of benzo(a)pyrene in ovarian and hepatic microsomes of rat, mouse, sheep, goat, pig, and cow. Values are expressed as mean concentration of total metabolites + SE ($n = 5$ for each species). Data from triplicate determination (variability was <10%) of five individual animals from each species were compared. Asterisks denote statistical significance ($P < 0.05$) in BaP metabolite concentrations between rat and the rest of the animal species.

Multiple species comparison of this data, performed by two-way ANOVA also revealed that the differences were statistically significant ($P < 0.001$) between rodents (rat and mouse) and higher mammals (ram, pig, and cow). Additionally, even among the higher mammals, in a majority of the cases, the differences in metabolite concentrations were different ($P < 0.001$) both in ovary and liver (Fig. 1). Interestingly, hepatic microsomes produced the greatest quantities of metabolites relative to that of ovarian microsomes in all the species studied ($P < 0.05$; Fig. 1). This observation is not surprising in as much as the liver functions as the main detoxifying organ in mammals (Wall et al., 1991). The variation in production of BaP metabolites by microsomes in animal groups used in this study could be attributed to differences in constitutive levels of drug metabolizing enzymes (Lewis et al., 1998). Also, induction of microsomal enzymes by dietary ingredients (Ioannides, 1999) especially in farm animals cannot be ruled out.

Since it is established that metabolism of PAHs in mammalian ovary is hormonally regulated (Bengtsson et al., 1987), we measured the BaP metabolite concentrations produced by microsomes isolated from prepubertal and cycling pigs and established that BaP metabolism was indeed influenced by stage of the sexual development. Microsomes isolated from gilts that were at mid-luteal and follicular phases metabolized BaP rapidly, compared to those in the prepubertal stage (Table I). Normally, one would expect to see increased concentrations of BaP metabolites in the ovaries of cycling than prepubertal pigs because the cycling animals secrete more ovarian steroids (estrogen and progesterone) by antral follicles of different stages of maturation and the corpora lutea (CL) (Archibong, 1987). On the contrary, the high concentrations of metabolites in luteal phase are not uncommon due to a high constitutive expression of CYP-regulated luteal estrogen. Our findings are in agreement with those of Eliasson et al. (1997) who reported that

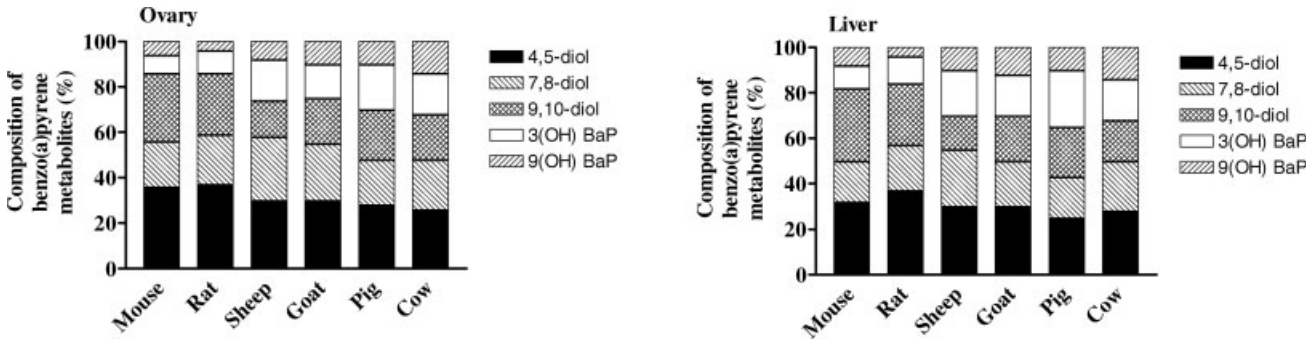

Fig. 2. Metabolite profiles of benzo(a)pyrene in ovary and liver microsomes of rat, mouse, sheep, goat, pig, and cow. Values are expressed as the percentage of individual metabolite types among the total metabolites (sum of individual concentrations of BaP 9,10-diol; BaP 4,5-diol; BaP 7,8-diol; 1-hydroxy, 3-hydroxy, and 9-hydroxy BaP) formed; $n = 5$ for each species.

TABLE I. Benzo(a)pyrene metabolite concentrations generated by microsomes from various stages of estrous cycle in pig

Ovarian Status	Description	BaP Metabolite Concentrations (pmoles/min/mg protein)
Prepubertal ($n = 6$)	Minimal estrogen stimulation of the uterus	$0.10 \pm 0.0001^{****}$
Prepubertal ($n = 4$)	Some uterine stimulation	$0.12 \pm 0.01^{***}$
Postpubertal ($n = 2$)	Follicular to late follicular phases, 14–15CA, many 4–5 mm follicles	$0.17 \pm 0.02^{**}$
Postpubertal ($n = 5$)	Mid-luteal, 11–17 CL	0.22 ± 0.02

CA, corpora albicans; CL, corpus luteum.
$^{****}P < 0.0001$; prepubertal (minimal estrogen stimulation) versus postpubertal (follicular and mid-luteal).
$^{***}P < 0.001$; prepubertal (some estrogen stimulation) versus postpubertal (follicular and mid-luteal).
$^{**}P < 0.01$; prepubertal (some estrogen stimulation) versus postpubertal (follicular).

metabolism of dimethylbenz(a)anthracene was highest in CL compared to preovulatory follicles. Furthermore, the higher concentrations of BaP metabolites in the CL, particularly the metabolites in the organic fraction (less polar) could be secondary to the higher lipid content of the CL.

Benzo(a)pyrene metabolites, being lipid soluble are transported across the cell membrane by passive diffusion (Castelli et al., 2002), enter the germ cells and may cause apoptosis (Mattison et al., 1985) leading to ovarian follicular atresia (Hsueh et al., 1994; Mann et al., 1999; Savabieasfahani et al., 1999). Information on the composition of metabolites that arise from bioactivation of BaP in the presence of microsomes is relevant in understanding the causal factors involved in toxicity. Initial oxidation of BaP catalyzed by CYP450 family of enzymes (CYP1A1, CYP1A2, and CYP1B1) yield arene oxides (9-OH-BaP, 7-OH-BaP, 6-OH-BaP, 3-OH-BaP, and 1-OH-BaP). These arene oxides rearrange to phenols or undergo hydration catalyzed by epoxide hydrolase (EH) generating BaP-9,10-diol; BaP-7,8-diol; and BaP-4,5-diol (reviewed in Ramesh et al., 2004; Shimada and Guengerich, 2006). Of the drug metabolizing enzymes that contribute to differential susceptibilities to the toxic effects of BaP, the CYP1A1 is not constitutively expressed in various species and CYP1A2 is mostly hepatic (Guengerich, 1997). Therefore, the biotransformation of BaP by ovarian microsomes could have been the result of CYP1B1 and EH that were reported to be constitutively expressed in ovarian tissues (DiBiasio et al., 1991; Otto et al., 1992). One of the products of BaP biotransformation, the BaP-7,8-diol is further oxidized to 7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE). This diol epoxide reacts with cellular macromolecules and cause toxicity (Xue and Warshawsky, 2005).

The BaP metabolites identified from both ovary and liver tissues were BaP 9,10-diol, BaP 4,5-diol, BaP 7,8-diol, 3-hydroxy and 9-hydroxy BaP. There were no remarkable differences between hepatic and ovarian microsomes in the BaP metabolite types formed. The rodent ovarian microsomes produced considerably higher proportion ($P < 0.05$; treatment × species interaction) of BaP 4,5-diol, and 7,8-diol than the other animal species in this study. However, hepatic microsomes from the latter animal groups converted a greater proportion than rodents ($P < 0.05$; treatment × species interaction) of BaP to 3- and 9-hydroxy BaP that are considered to be a part of the detoxification pathway.

Using estrogen receptor binding assays BaP/metabolites were estrogen antagonists (Arcaro et al., 1999). Furthermore, *in vivo* studies conducted in our laboratory have revealed that sub acute exposure to BaP resulted in antiestrogenic activities (Archibong et al., 2002) that are driven by BaP metabolism. Interestingly, the microsomal CYP families of enzymes that generate these metabolites in liver and extra hepatic tissues such as the ovary are also involved in steroid hormone metabolism (Jefcoate et al., 2000). Upon prolonged exposure to BaP, sequestration of this chemical in high-density lipoproteins (Polyakov et al., 1996) that are essential for steroid hormone biosynthesis in the ovary cannot be ruled out. This can lead to reduced secretion of gonadotropins such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) at proestrus in lower mammalian species and at ovulatory phase of the menstrual cycle of women with adverse outcomes in the final stages of follicular development (Neal et al., 2007). Extrapolation of *in vitro* data to *in vivo* situation has some limitations in that, especially in farm animals, the likelihood of more amounts of biotransformation enzymes in one species and an intrinsically greater rate of clearance cannot be ruled out. Toward this end, our long-term goal is to study the biotransformation enzyme activities, expression and kinetic parameters, and the inhibition profiles for ovarian microsomes of each species and compare those obtained for human ovarian microsomes to assess the animal species most similar to humans who are exposed to PAHs via cigarette smoke, occupational settings, and diet.

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