MICROSOME-MEDIATED BENZO[A]PYRENE-DNA BINDING AND INHIBITION BY CYTOSOLIC FRACTIONS FROM LIVER AND SKIN OF ADULT AND WEANLING RATS

M. Mohammadi Qanatestani[1], A. Allameh[2] and M. R. Noori-Daloii[3*]

[1] *Department of Basic Sciences, Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Islamic Republic of Iran*
[2] *Department of Biochemistry, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Islamic Republic of Iran*
[3] *Department of Medical Genetics, Faculty of Medicine, Tehran University of Medical Sciences, Islamic Republic of Iran*

Abstract

Biotransformation of benzo[a]pyrene (BaP) in the presence of microsomal fractions derived from liver and epiderm of adult and weanling rats was examined. The aim of this study was to evaluate the effect of age on the capacity of two organs in transformation of BaP. Subcellular fractions were prepared from skin and liver by ultracentrifugation and were used as the source of BaP metabolizing enzymes in a reconstitution assay system. Microsomal fractions are sources of cytochrome P-450 and cytosols are the source of glutathione S-transferase (GST). In a metabolic activation assay system, cytochrome P-450 catalyses the formation of reactive epoxide of BaP which can then interact with exogenous DNA. Adult rat liver microsomes with the highest cytochrome P-450 and maximum capacity for BaP-DNA adducts formation (~204 pmol BaP bound/mg DNA) are considered as positive control in this assay system. The adduct formation in the presence of adult and young rats was approximately 204 and 27 pmol/mg DNA, respectively. Microsomes prepared from skin samples of adult and young rats mediated approximately 49 and 16 pmol BaP binding to DNA respectively. With the addition of cytosol to the microsome-mediated system an *in vitro* detoxification model has been established. The results obtained by the addition of different cytosolic samples showed that liver cytosol which contains highest GST activity caused about 28% inhibition in BaP binding to DNA. The inhibitory effects of cytosolic fraction from weanling liver, adult skin and weanling skin were 17, 19 and ~9% respectively. These data show that isolated subcellular fractions from young rats are less efficient in the biotansformation of BaP. However, the results obtained *in vitro* do not reflect the changes *in vivo*. Further, *in vivo* experiments should be carried out after BaP administration to animals to confirm the differences in the BaP-DNA adduct formation and BaP-glutathione conjunction in tissues of young and adult animals.

Keywords: Benzo[a]pyrene; Microsome; Cytosol; Liver; Skin; DNA adducts; Weanling rat

Introduction

Benzo[a]pyrene (BaP) in the ubiquitous polycyclic aromatic hydrocarbon (PAH) implicated in cancer of several organs particularly skin and lung. Certain working environments, diet and cigarette smoke are major sources of BaP [1-3].

BaP like many other chemical carcinogens must be

* *E-mail: nooridaloii@excite.com*

metabolically activated in the cell in order to exert its mutagenic and carcinogenic effects. After absorption and distribution in the body, BaP is metabolically activated by cytochrome P-450 to various metabolites. BaP-7,8 diol has been identified as the proximate carcinogen and BaP-epoxide i.e. BaP-7,8-diol-9,10-epoxide (anti-BPDE is known to be the ultimate carcinogenic form of BaP [4,5]. BPDE binds covalently to cellular macromolecules such as proteins, RNA and DNA. BaP adduct formation to nuclear DNA is the initiation step involved in carcinogenesis [6,7].

The amount of carcinogen-DNA adducts detected is dependent on several physiological factors including carcinogen exposure, the metabolic balance between carcinogen activation and detoxification and DNA repair capacity. The metabolic activation and detoxification of BaP by various cell types in different tissues leads to tissue-specific differences in the rate of activation, detoxification and the relative proportions and total amounts of metabolites produced. The biological effects of BaP depend on several factors such as the animal species, genetic factors, sex and the target organ [1]. The activity of both phase I and phase II drug metabolizing systems in liver of rats have been observed to be gradually increased with age. Differences in the drug metabolizing factors during development has been reflected in the metabolic activation and glutathione conjugate formation catalysed by glutathione S-transferase (GST) with other chemical carcinogens [9].

Alterations in cytochrome P-450 isoenzymes due to physiological factors such as differences in organs and age-related changes are important factors which determine the carcinogenicity of BaP. Similarly the activity of enzymes and substrates involved in phase II drug metabolism (detoxification pathways), particularly glutathione conjugation pathways plays a major role in the carcinogenesis of BaP [10,11]. Growing rats like adults are equally exposed to the environmental PAHs but it is generally believed that the xenobiotic metabolizing system is underdeveloped during the early stages of life. In this study using *in vitro* approaches (cell free extract) we have made attempts to compare the efficiency of tissues of an adult and weanling rat in activation and inactivation of BaP.

Materials and Methods

Chemicals

Calf thymus DNA, bovine serum albumin (BSA), EDTA, dimethyl-sulfoxide (DMSO), benzo[a]pyrene, reduced glutathione (GSH) and NADPH were purchased from Sigma Chemical Co. St. Louis, Mo. USA. Radiolabeled BaP (specific activity 90 µCi/mmol) was the product of Amersham Corporation. UK. All other chemicals and reagents used were from E. Merck, Germany.

Animals

Young adult female rats of Wistar strain weighing 200±15 g and recently weaned rats (one-week old) were used for preparation of subcellular fractions. Breeding and raising of the rats were carried out under controlled temperatures and a 12 h light/dark cycle. Animals were maintained on a standard commercial pellet and had free access to food and water.

Isolation of Subcellular Fractions

In order to prepare microsomes and cytosols from liver and epiderm, first the back of the animals was shaved with an electric clipper. Adult and young rats were sacrificed by cervical dislocation and a portion of the epiderm on the shaved area was separated. Livers of rats in both of the age-groups were also removed and immediately transferred into a beaker containing homogenizing buffer. Connective tissues were dissected out from the livers and epiderm. The tissues were homogenized with the help of a polytron homogenizer and processed for isolation of subcellular fractions by ultracentrifugation as described earlier [12]. Microsomes obtained were used as a rich source of cytochrome P-450 and cytosols were used as the source of glutathione S-transferase.

In vitro Incubation System

This incubation system is designed to examine the efficiency of microsomal cytochrome P-450 (microsomes obtained from different sources) in catalyzing the conversion of BaP to its epoxide form which readily interacts with nucleophilic centers such as DNA. The assay was carried out in duplicate in test tubes. Each tube contained 1.5 ml of phosphate buffer, (100 mM, pH=7.4). The incubation mixture consisted of microsomes equivalent to 1 mg protein, 0.5 mg calf thymus DNA, 1 mM NADPH, 3 mM $MgCl_2$ and 80 nM of radiolabelled BaP (specific activity 70 Ci). The final volume was 2.0 ml and the reaction was carried out at 37°C for 90 min. After the incubation, DNA was isolated by phenol extraction procedure. An equal volume of the extraction mixture containing phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) was added to the mixture and DNA extraction was performed as described by Chang and Bjeldance [13]. The amount of DNA extracted was determined spectrophotometrically at 260 nm and based on the A260/280 nm ratio of about 1.8. Radiolabeled DNA samples were dissolved in 2 ml of Tris-EDTA buffer and mixed with toluene-based scintillation liquid for determination of the amount of BaP bound to DNA.

Inhibition of BaP-DNA Adduct Formation

Addition of cytosolic fractions as the source of GST

to the incubation system resulted in the inhibition of the BaP-DNA adduct formation. The activity of the cytosolic GST varies depending on the source of the cytosol. The rate of DNA binding in a reaction mixture containing adult rat liver microsomes without any cytosol was considered as 100%. The amount of BaP-DNA inhibition in the presence of cytosolic fractions obtained from other tissue sources were calculated accordingly.

Other Assays

Protein concentration was determined in subcellular fractions by the method of Bradford [14] using bovine serum albumin (BSA) as a standard. Microsomal cytochrome P-450 content was measured by the method of Omura and Sato [15].

Results

The results of microsome-mediated BaP binding to calf thymus DNA are summarized in Fig. 1. Age-related differences in the catalytic activity of hepatic microsomal cytochrome P-450 showed that BaP-DNA adduct formation in the presence of adult liver microsomes is much higher as compared with that of weanling rats (Fig. 1). The amount of BaP bound to calf thymus DNA in the presence of liver microsomes derived from adult and young rats were calculated to be 204 and 27 pmol/mg DNA, respectively. Figure 1 further describes that the adult skin has relatively more capacity than that of young rats to epoxidize BaP as judged by its binding to calf thymus DNA. BaP-DNA binding in presence of adult and young skin microsomes was 49 and 16 pmol/mg DNA, respectively (Fig. 1). Such differences could be attributed to the cytochrome P-450 content of the microsomes as shown in Figure 2.

The results of the inhibitory effects of cytosolic fraction (source of GST) obtained from either skins or livers of the two age-groups are presented in Fig. 3. the degree of binding in control groups (without cytosol) was considered as 100% and the percentage of inhibition in the presence of cytosolic fraction was calculated accordingly. The data obtained from this assay system showed that GST activity in cytosolic fraction determines the rate of BaP-DNA modulation. Adult liver cytosol and cytosol prepared from weanling skin caused maximum (~28%) and minimum (~8.7%) inhibition in the adduct formation respectively.

Discussion

Benzo[a]pyrene like several other chemical carcinogens is a procarcinogen that undergoes biotransformation in the cells and is converted to its ultimate carcinogenic form. Biotransformation of BaP including phase I (metabolic activation) and Phase II (inactivation) are two sequential and compulsory steps

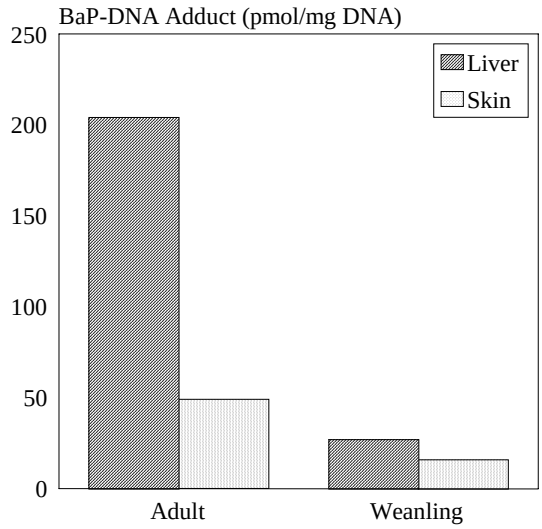

Figure 1. Microsome-dependent Benzo[a]pyrene binding to exogenous DNA.

Preparation of microsomes and incubation system is as described under “Materials and Methods” section. Data are the average values of 5 analysis carried out in duplicate. DNA was extracted immediately after incubation. Radioactive BaP bound covalently to DNA was estimated by counting radioactivity in DNA samples and expressed as pmol carcinogen bound/mg DNA/90 minutes.

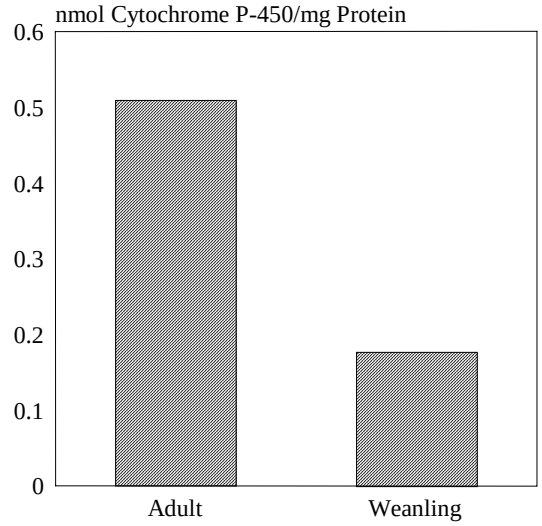

Figure 2. Comparison of cytochrome P-450 content of microsomes prepared from livers of young and adults.

The details about microsome preparation and cytochrome P-450 estimation are as described in “Materials and Methods”.

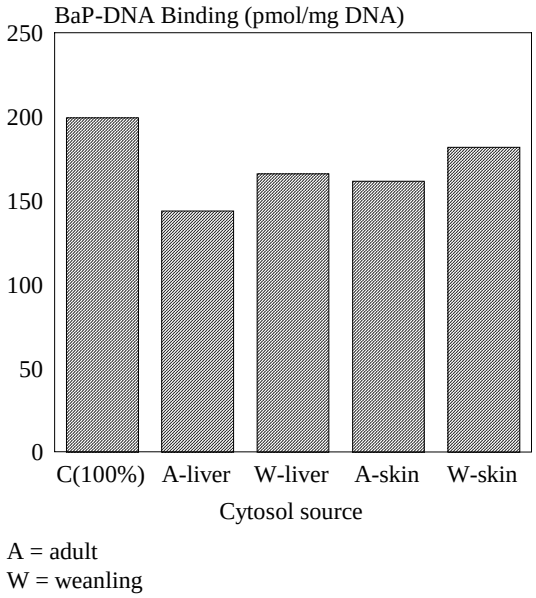

Figure 3. Modulations of BaP-DNA adduct formation by cytosolic fraction prepared from liver and skin.

Incubation mixture is as described under "Materials and Methods". In this assay the source of microsomes was adult rat liver. Cytosolic fraction prepared either from skin or liver samples equivalent to 30 mg wet tissue was added to each tube. Results are average of 5 analyses carried out in duplicate. In the control group no cytosol was added and was considered as 100% binding.

for the elimination of BaP toxic metabolites. Phase I metabolites on one hand are dangerous since they can readily attack nucleophilic centers in cells such as DNA, and on the other hand these metabolites are good substrates for phase II enzymes.

Metabolic activation of BaP is catalysed by cytochrome P-450 system while inactivation of these metabolites is performed by conjugation pathways. Among conjugation pathways, glutathione conjugation of BaP is the predominant pathway for detoxification of the reactive epoxide of BaP.

There are several physiological factors which can alter the rate of the carcinogen biotransformation. Animal species, genetic factors, sex, age and environmental agents are major factors which determine the carcinogenicity of BaP. Organ-specific differences in the key enzymes involved in xenobiotic metabolism are also important factors contributing to the effects of BaP. In the present communication in a reconstitution system we have compared the efficiency of microsomes derived from different sources to activate BaP. In this system exogenous DNA was used to trap the epoxide formed. The advantage of this system is that the interference from other molecules in the system is minimized. The rate of microsome-mediated BaP-DNA adduct formation was dependent on the source of the microsomes. Comparative studies with skin and liver samples from young and adult rats revealed that the amounts of BaP-DNA adduct formation is highest i.e. 204 pmol BaP bound /mg DNA in the system mediated by adult liver microsomes. In contrast, the capacity of weanling rat skin microsomes to activate BaP was minimum i.e. 16 pmol BaP bound/mg DNA. These data show that when the carcinogen is absorbed from skin epidermal, cells can locally metabolize a portion of BaP and probably part of it is translocated to the liver where it is the main site for xenobiotic metabolism and a rich source of cytochrome P-450. Differences in the microsomal content of adult and growing rat livers are also considerable. Adult rat hepatic cytochrome P-450 is about 8 folds greater than that of weanling rats (Fig. 2). Microsomal cytochrome P-450 content is well reflected in our reconstitution assay system. Figure 1 shows that liver microsomes obtained from adult and weanling rats catalyse the binding of approximately 204 and 27 pmol BaP/mg DNA, respectively. Previously, similar results were recorded using aflatoxin B1 as the carcinogen in an *in vitro* system [16]. Developmental changes in phase I enzymes, is in consistence with glutathione conjugation system. Figure 3 shows that the inhibitory effect of cytosolic fraction purified from various sources on BaP-DNA adduct formation is different. Addition of the cytosolic fractions from liver of adult or growing rats to the incubation system caused ~28 and ~17% inhibition in BaP-DNA binding, respectively. Cytosolic fraction from epiderm of adult and growing rats caused BaP-DNA inhibition to a lesser extend (adult skin cytosol; ~19% and weanling skin cytosol; 8.7%). It is interesting to note that despite the lower activities of phase I and phase II drug metabolizing systems in growing tissues, *in vivo* experiments carried out in liver, lungs and kidneys of the young rats treated with aflatoxin B1 show that newborn and weanling rats are capable of handling xenobiotics and protect their cellular macromolecules against such toxins. It is generally believed that young laboratory animals are less resistant to the toxic effects of chemical carcinogens [9]. This conclusion is derived mostly from the experiments carried out *in vitro* and based on the estimation of enzymes in isolated subcellular fractions. However, there are several other factors *in vivo* which are involved in the metabolism and detoxification of a carcinogen and its metabolites. Newborn and weanling rats probably use rare and alternative mechanisms to compensate the deficiencies in drug metabolizing factors. This could also be explained by performing experiments in young rats pretreated with carcinogens. *In vivo* resistance of young rats after drug administration could be attributed to the expression of highly inducible GST isoenzymes. Such enzymes are induced readily in

tissues of young animals as a temporary response to the xenobiotics [16]. In addition to the inducible GST isoenzymes, the contribution of weanling rat kidneys in formation of glutathione conjugate and elimination of toxic metabolites is also an important protective mechanism [17].

References

1. Pelkanon, O. and Nebert, D. W. Metabolism of polycyclic aromatic hydrocarbons. Etiologic role in carcinogenesis. *Pharmacol. Rev.,* **34**, 189-222, (1982).
2. Miller, J. A. Carcinogenic be chemicals: An overview. GHA clowes memorial lecture. *Cancer res.,* **30**, 559-76, (1970).
3. Bartsch, H. DNA adducts in human carcinogenesis: etiological relevance and structure-activity relationship. *Mutation Res.* **340**, 67-69, (1996).
4. Philips, D. H. and Sims, P. Polycyclic aromatic hydrocarbon metabolites. Their reaction with nucleic acid. In: Grover, P. L. (Ed.), Chemical Carcinogens and DNA. CRC Press. Boca Raton. FL. Vol. 2, pp. 29-57, (1979).
5. Philips, D. H., Grover, P. L. and Sims, P. The covalent binding of polycyclic aromatic hydrocarbons to DNA in the skin of mice of different strains. *Int. J. Cancer,* **22**, 487-94, (1978).
6. Melikian, A. A., Bagheri, K. and Hechts, S. S. Contrasting disposition and metabolism of topically applied benzo[a]trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene and 7B,8α-dihydroxy-9α,10α epoxy-7,8,9,10 tetrahydrobenzo[a]pyrene in mouse epiderm *in vivo. Cancer Res.,* **47**, 5354-66, (1987).
7. Bjelogrlic, N., Iscan, Raunio, H., Pelkanon, O. and Vahakangas, K. Benzo[a]pyrene-DNA adduct and monooxygenase activities in mice treated with benzo[a]pyrene, cigarette smoke and smoke condensate. *Chem. Biol. Interactions.* **70**, 51-61, (1989).
8. Brauze, D., Mikstacka, R. and Baer-Dubowska, W. Formation and persistence of benzo[a]pyrene-DNA adduct in different tissues of C57BL/10 and DBA/2 mice. *Carcinogenesis,* **12**, 1607-11, (1991).
9. Behroozikha, M., Saeedi, M. and Allameh, A. Comparison of aflatoxin B1-DNA binding and glutathione conjugate formation by liver preparation from rats of different ages. *Cancer Lett.,* **66**, 69-76, (1992).
10. Alexandrov, K. and Rajan-Morano, M. *In vivo* DNA adduct formation by Benzo[a]pyrene in mouse and rat epidermal and dermal fibroblasts after topical application of an initiating dose of Benzo[a]pyrene. *Arch. Goschwalsforsch,* **60**, 329-40, (1990).
11. Nomatan, N. Glutathione, glucuronide and sulfate transferase in polycyclic aromatic hydrocarbon metabolism. In: Polycyclic hydrocarbons and cancer. Gelboin H. V. and Tso (Eds.). Academic Press, New York, Vol. 3, pp. 213-58, (1981).
12. Allameh, A., Saxena, M. and Raj, H. G. Differential effects of butylated hydroxy-anisole on metabolism of aflatoxin B1 *in vitro* by liver and lung microsomes. *Cancer Lett.,* **40**, 49-57, (1988).
13. Chang, Y. and Bjeldanes, L. F. R. Goitrin and BHA-induced modulation of aflatoxin binding to DNA and biliary excretion of thiol conjugates in rats. *Carcinogenesis,* **8**, 585-90, (1987).
14. Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-57, (1976).
15. Omura, T. and Sato, R. The carbon monoxide binding pigment of liver microsomes. Evidence for its hemoprotein. *J. Biol. Chem.,* **239**, 2370-78, (1964).
16. Allameh, A., Nikseresht, M. and Kheyrdoosh, F. Role of cytosolic glutathione S-transferases in protection against acetaminophen-induced lipid peroxidation in weanling rats. *Medical J. I. R. Iran,* **13**, 213-17, (1999).
17. Allameh, A., Farahani, M. and Zarghi, A. Kinetic studies of aflatoxin B1-glutathione conjugate formation in liver and kidneys of adult and weanling rats. *Mechanism of Ageing & Development,* **115**, 73-83, (2000).