Environmental Health Perspectives
Vol. 62, pp. 223-226, 1985

Examination of Adduct Formation *In Vivo* in the Mouse Between Benzo(a)pyrene and DNA of Skin and Hemoglobin of Red Blood Cells

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We are interested in devising techniques which will allow us to measure and quantitate exposure to chemical carcinogens and which eventually can be used in risk analysis with humans. Our recent research with HPLC/fluorescence has demonstrated that we can detect, identify, and quantitate the binding of benzo(a)pyrene (BaP) with DNA of mouse skin. The technique not only allows femtomole amounts of BaPDE associated with DNA isolated from a single mouse skin to be detected using conventional instrumentation, but also establishes the stereochemical origin of the adduct, and has been employed in the investigation reported here to estimate the concomitant binding of BaP to hemoglobin *in vivo*.

The temporal existence of BaPDE/DNA adducts in mouse skin over a 5-week period showed that at 35 days after treatment, approximately 15% of the initial adducts were still detectable even though DNA turnover would predict that they should have been deleted from the genome. The concentration of the major covalently bound adduct, anti-BaPDE/deoxyguanosine, relative to the total BaPDE/DNA adduct population remained essentially constant during the 5-week period.

It is known that topically applied BaP is absorbed, metabolized, and excreted by the mouse. Examination of hemoglobin of mouse RBCs 24 hr after BaP treatment revealed covalent adduct formation exclusively via anti-BaPDE. The dose response of adduct binding to hemoglobin and DNA appeared to be similar.

Introduction

Many chemical carcinogens, among them the polycyclic aromatic hydrocarbons, have been shown to bind covalently to cellular macromolecules after they undergo metabolic activation (*1–3*). Furthermore, the tumorigenic and carcinogenic activities of these compounds, and in particular benzo(a)pyrene (BaP) have been correlated with their ability to form adducts with DNA (*4,5*).

We are interested in devising techniques which will allow us to detect and quantitate exposure to chemical carcinogens and which eventually can be used in risk analysis with humans. Our recent research with HPLC/fluorescence has demonstrated that we can detect, identify, and quantitate the binding of BaP with DNA of mouse skin (*6–8*). The technique not only allows femtomole amounts of BaPDE associated with DNA isolated from a single mouse skin to be detected using conventional instrumentation, but also establishes the stereochemical origin of the adduct.

In the investigation reported here we show a correlation between the degree of adduct formation in the DNA of the skin and the extent of alkylation of hemoglobin after exposure of the mouse to BaP.

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Methodology

Sample preparation: BaP in acetone, or acetone alone, was applied topically to the dorsal skin of C3H mice. Periodically thereafter, the animals were sacrificed, the dorsal skin removed, and blood was drawn into heparinized tubes.

BaP Binding to DNA

The protocol for the estimation of BaP binding to mouse skin DNA was as follows: (1) proteolytic digestion of the tissue; (2) deproteinization and partitioning of nucleic acids with chloroform/phenol; (3) enzymatic depolymerization of RNA and spermine precipitation of DNA; (4) BaPDE release from DNA in form of tetrol by acid hydrolysis; and (5) tetrol resolution by HPLC and quantitation by fluorescence analysis.

BaP Binding to Hemoglobin

The protocol for the estimation of BaP binding to hemoglobin of mouse erythrocytes was as follows: (a)

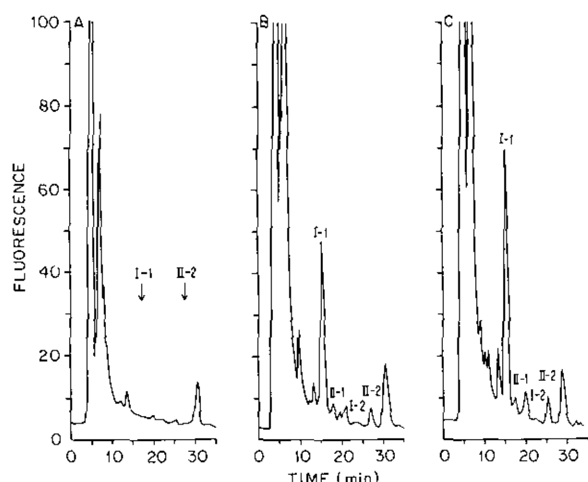

FIGURE 1. HPLC/fluorescence profiles of tetrols: (*A*) release of acid-hydrolyzed DNA from skin of mouse that received acetone alone; (*B*) acid-hydrolyzed calf thymus DNA treated *in vitro* with authentic *anti-* and *syn*-diol epoxides of BaP; (*C*) acid-hydrolyzed DNA from dorsal skin of mouse exposed to 200 μg BaP in acetone.

recovery of RBCs and lysis with water; (2) removal of cell debris; (3) isolation of globin by precipitation with acetone/5mM HCl where noted; (4) and (5) as in the DNA protocol.

Results

BaPDE–DNA Adducts

Figure 1 shows typical fluorescence profiles obtained by HPLC/ODS chromatography for various materials tested. Figure 1*A* is the fluorescence profile of acid-hydrolyzed DNA from dorsal skin of a mouse that had acetone topically applied. No fluorescent peaks indicative of tetrols are observed. Figure 1*B* is the profile derived from the hydrolysis of calf thymus DNA treated *in vitro* with authentic *anti-* and *syn*-diol epoxides of BaP. Figure 1*C* is the profile of acid-hydrolyzed DNA obtained from the dorsal skin of a mouse exposed to BaP for 24 hr.

Persistence of Anti-BaPDE–DNA Adducts

The temporal existence of BaPDE–DNA adducts in C3H mouse skin is illustrated in Figure 2. Thirty days after a single application of BaP, a marked decrease in adducts has occurred. A small percentage of the total population of adducts continue to persist after 4 weeks with a half-life approaching 30 days. The fraction of anti-BaPDE–deoxyguanosine adducts, which represents the major covalently bound deoxynucleoside (*3*) present in the total population of anti-BaPDE–DNA adducts was determined. The data obtained indicate no preferential removal or persistence of the anti BaPDE–deoxyguanosine adduct with respect to the total BaPDE–DNA adduct population over the time span shown in Figure 2.

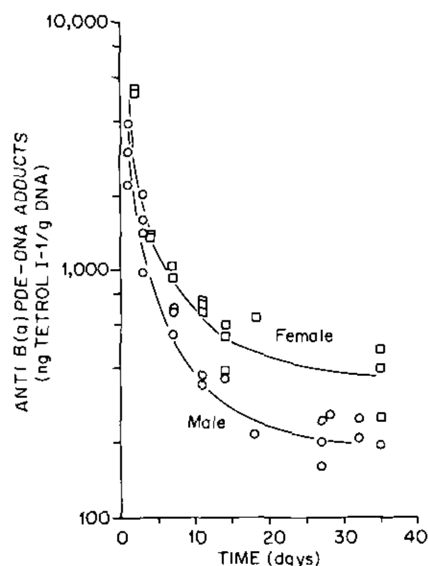

FIGURE 2. Time course of anti-BaPDE binding (or tetrol I-1) to skin of C3H (○) male or (□) female mouse. Mice were treated topically with 200 μg of BaP in acetone and DNA isolated at various times from 1 to 35 days after treatment.

BaP Binding to DNA and Hemoglobin

Figure 3 is the HPLC/fluorescence profiles of tetrols which were acid-released (*A*) from mouse skin 24 hr after exposure to a single topical dose of BaP, and (*B*) from hemoglobin isolated from RBCs of the same mouse. These data show that BaP forms adducts not only with the DNA in the target tissue, but also concomitantly with the hemoglobin. The absence of tetrols II-1 and II-2 in the HPLC/fluorescence chromatography of hemoglobin indicates that adduct formation in the RBC is almost exclusively via the anti-BaPDE metabolite of BaP.

Globin as Target Molecule in RBCs

That globin is the molecule in the RBCs where adduct formation occurs is inferred from the following observations. Upon lysis of the RBCs, no BaP binding is found in the cell debris. The degree of binding found in the recovered hemoglobin does not change upon extraction of ethyl acetate, indicating that the tetrols released by acid hydrolysis are from covalently bound BaP metabolites. Furthermore, globin isolated by treating the hemoglobin with acetone/5 mM HCl at −20°C still contains BaP adducts which are unaffected by subsequent treatment with RNase and DNase, but which become labile to proteolytic activities.

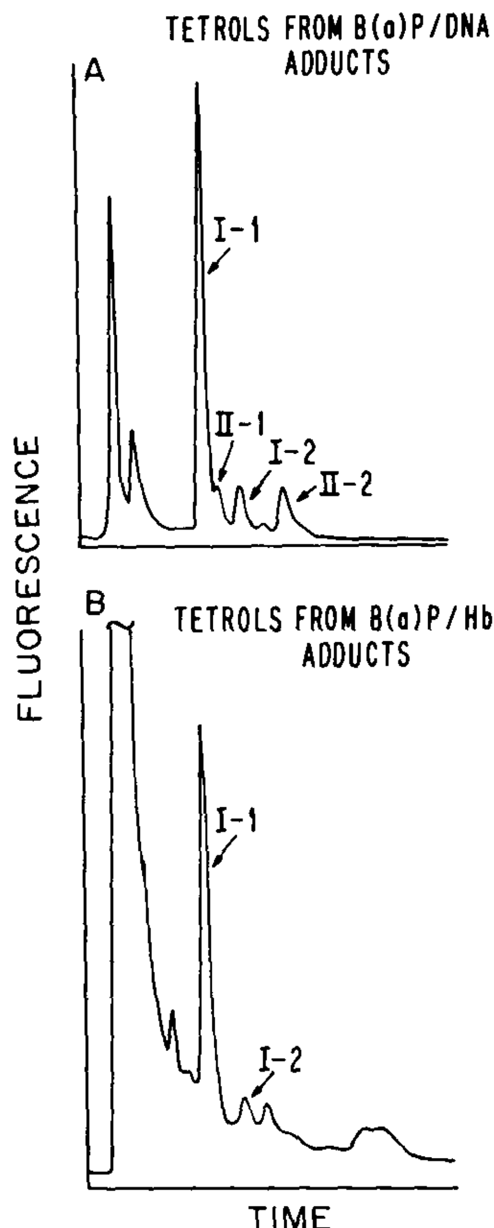

FIGURE 3. HPLC/fluorescence profiles of tetrols: (*A*) liberated from DNA isolated from female, C3H mouse skin 24 hr after exposure to a single topical dose of 300 μg BaP; (*B*) liberated from hemoglobin isolated from RBCs of same mouse.

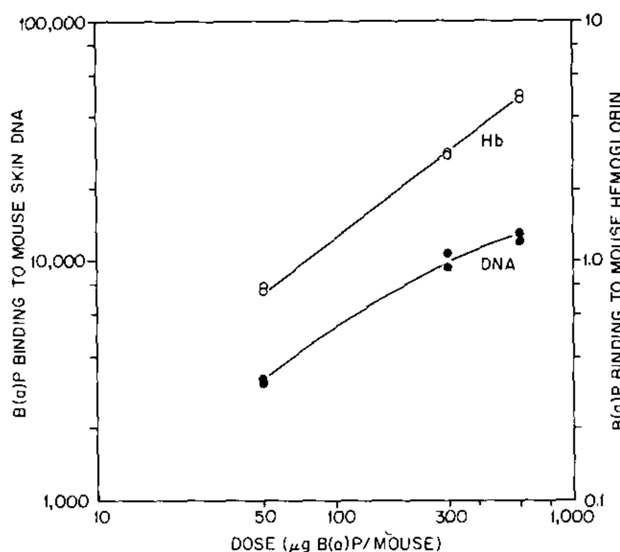

FIGURE 4. Log–log plot relating BaP binding versus the dose of BaP applied to mouse skin: (○) BaP binding of hemoglobin expressed as pg tetrol I-1/mg hemoglobin; (●) BaP binding to DNA expressed as ng tetrol I-1/g DNA. Each data point represents the average values from two mice.

Similarity between Adduct Formation with DNA and Hemoglobin

The amount of adduct formation in both DNA and hemoglobin was determined in a group of mice 24 hr after being exposed topically to various concentrations of BaP. The data are plotted as the log of BaP binding observed versus the log dose of BaP applied (Fig. 4). The striking feature of these data is the apparent parallelism of the two curves.

Discussion

It is known that topically applied carcinogens, such as PAHs, are absorbed, metabolized, and excreted by the mouse (*9,10*). In the cells of the skin at the site of application and where subsequent tumor formation occurs, metabolites of PAHs are converted to electrophilic agents which react with nucleophilic centers in nucleic acids and proteins (*1–3*). A good correlation has been demonstrated between the amount of BaP bound to the DNA of mouse skin and its carcinogenic activity (*4,5*). In fact, the ultimate carcinogenic form is thought to be the BaP-metabolite, anti-BaPDE, and it has been proposed that the quantitation of adduct formation of this compound with DNA be used as a measure of the "biologically effective" dose for BaP carcinogenicity (*11,12*).

We have shown a correlation between the degree of adduct formation in the target organ and the extent of alkylation of hemoglobin with benzo(a)pyrene, a classical PAH.

The suitability of hemoglobin as a cellular molecule for estimating carcinogenic risk of BaP exposure (and possibly PAHs) is suggested by the data presented here. First, the interaction of BaP with hemoglobin is highly specific, and occurs, presumably, via anti-BaPDE, the same ultimate carcinogenic form of BaP which interacts with DNA in the target tissue; second, the dose response of anti-BaPDE binding to hemoglobin appears similar to that of DNA, thus reflecting the "*in vivo*" carcinogenic dose of BaP; third, the adduct, once formed, is easy to measure; and fourth, hemoglobin is readily available.

Research sponsored by the Office of Health and Environmental Research, U. S. Department of Energy, under Contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.

REFERENCES

1. Gelboin, H., and T'so, P. O. P. (Eds.). Polycyclic Hydrocarbons and Cancer, Molecular Biology and Environment. Academic Press, New York, 1978.
2. Harvey, R. Polycyclic hydrocarbons and cancer. Am. Scientist 70: 386–393 (1982).
3. Philips, D., and Sims, P. PAH metabolites: their reaction with nucleic acids. In: Chemical Carcinogens and DNA, Vol. 2 (P. L. Grover, Ed.), CRC Press, Inc., Boca Raton, FL, 1979, pp. 9–57.
4. Brookes, P., and Lawley, P. Evidence for the binding of polynuclear aromatic hydrocarbons to nucleic acids of mouse skin: relationship between carcinogenic power of hydrocarbons and their binding to DNA. Nature 202: 781–784 (1964).
5. Weinstein, I. B. Current concepts on mechanism of chemical carcinogenesis. Bull. N. Y. Acad. Med. 54: 336–383 (1978).
6. Rahn, R., Chang, S., Holland, J., and Shugart, L. A fluorometric-HPLC assay for quantitating the binding of BaP metabolites to DNA. Biochem. Biophys. Res. Commun. 109: 262–268 (1982).
7. Shugart, L., Rahn, R., and Holland, J. Quantifying BaP binding to DNA by fluorescent analysis. In: PAH's: 7th International Symposium on Formation, Metabolism, and Measurement (M. Cooke and A. Dennis, Eds.), Battelle Press, Columbus, OH, 1982, pp. 1087–1097.
8. Shugart, L., Holland, J., and Rahn, R. Dosimetry of PAH skin carcinogenesis: covalent binding of BaP to mouse epidermal DNA. Carcinogenesis 4: 195–198 (1983).
9. Katin, P., Falk, H. L., and Busser, R. Distribution, retention, and elimination of C^{14}-3,4-benzpyrene after administration to mice and rats. J. Natl. Cancer Inst. 23: 541–555 (1959).
10. Sanders, C. L., Skinner, C., and Gelman, R. Percutaneous absorption of [7,10-^{14}C] BaP and [7,12-^{14}C] dimethylbenz[a]anthracene in mice. Environ. Res. 33: 353–360 (1984).
11. Perera, F., Poirier, M., Yuspa, S., Nakayama, J., Jaretzki, A., Curnen, M., Knowles, D., and Weistein, I. B. A pilot project in molecular cancer epidemiology: determination of BaP-DNA adducts in animal and human tissues by immunoassays. Carcinogenesis 3: 1405–1410 (1982).
12. Hoel, D., Kaplan, N., and Anderson, M. Implication of nonlinear kinetics on risk estimation in carcinogenesis. Science 219: 1032–1037 (1983).