Dose Response for Benzo(a)pyrene Adducts in Mouse Epidermal DNA¹

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ABSTRACT

The dose dependency of the binding of benzo(a)pyrene (BaP) with DNA of mouse epidermis was investigated. BaP-conjugated epidermal DNA was isolated and enzymatically degraded to deoxyribonucleosides. The BaP-DNA adducts were separated by Sephadex LH-20 column or high-performance liquid chromatography. Two major BaP-DNA adducts were found. One was in the region of the elution profile that contained poly cyclic aromatic hydrocarbons added to deoxyribonucleosides. The other adduct was eluted from Sephadex LH-20 and high-performance liquid chromatography columns before the deoxyribonucleosides and after deoxyribonucleotides. Both adducts of BaP in epidermal DNA reached a maximum 7 hr after a single skin application, and subsequently little, if any, loss of adducts was observed for 49 hr. Both adducts varied as a linear function for topical doses in the range from 0.01 to 300 μg/mouse. The formation of DNA adducts by BaP occurred in proportion to dose at doses several orders of magnitude below those that are feasible to test for carcinogenicity.

INTRODUCTION

When BaP⁴ is applied to mouse skin (6, 8, 13), added to cell cultures (6, 9), or incubated in the presence of microsomes and DNA (7), electrophilic metabolites are formed that covalently bind to DNA. The initiation of carcinogenesis by poly cyclic aromatic hydrocarbons is believed to involve covalent binding to cellular macromolecules, and there appears to be an especially good correlation between their carcinogenic potency and DNA binding (4). Thus, the binding to DNA might serve as a sensitive indicator of carcinogenicity since analytical techniques are available to detect such binding at extremely low doses. Actually, the formation of a number of different DNA adducts as well as tritium exchange onto the bases is possible. Thus, specific BaP-DNA adducts could serve as a better marker for biological potency than the total amount of binding in BaP-conjugated DNA. The major hydrophobic BaP adduct to deoxyribonucleosides formed in vivo has been identified and studied extensively (8, 10, 11, 14, 16, 21, 23, 27). Hydrophilic BaP-DNA adducts present in the elution profiles obtained from the chromatography systems used in the isolation of BaP:deoxyribonucleoside adducts remain to be characterized (17–20). The dose relationship and persistence of BaP adducts to epidermal DNA is reported here.

MATERIALS AND METHODS

Chemicals. [G⁻³H]BaP (40 Ci/mmole) and [¹⁴C]BaP (60.7 mCi/mmol) were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). BaP was obtained from Eastman Kodak Co. (Rochester, N. Y.). Calf thymus DNA, type 5; 3-methylcholanthrene; Coomassie Brilliant Blue G-250; 3,5-diamino-benzoic acid; DNase I (bovine pancreas); phosphodiesterase, type 2 (Crotalus adamanteus); and alkaline phosphatase, type 3 (Escherichia coli) were purchased from Sigma Chemical Co. (St. Louis, Mo.). RNase A was purchased from Worthington Biochemical Corp. (Freehold, N. J.) and heated at 90° for 10 min to destroy any DNase. Methanol distilled in glass was purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.).

[¹³H]BaP Binding to Mouse Epidermal DNA. Female HA/ICR mice were obtained from Sprague-Dawley (Madison, Wis.). The mice were 7 to 8 weeks old and in the resting phase of the hair cycle when [¹³H]BaP dissolved in 0.2 ml acetone was applied topically by pipet to the dorsal skin. The hair was removed with electric clippers 2 days before the applications, which were made between 7 and 9 a.m. The mice were killed by cervical dislocation. The dorsal skin was excised, placed on a paper card, and immediately immersed in liquid nitrogen. The epidermis was scraped from the frozen skin by means of a scalpel and homogenized in a Potter-Elvehjem homogenizer in a solution containing 1% sodium triisopropylphosphinate, 8% isopropyl alcohol 6% sodium p-aminosaliclyate, and 1% sodium chloride in water. The DNA was extracted by the Kirby phenol procedure as described by Diamond et al. (5).

Microsome Catalyzed DNA Binding of [¹³H]BaP. Mice were pretreated i.p. with 3-methylcholanthrene (2 mg/0.5 ml corn oil) and fasted 24 hr prior to sacrifice by cervical dislocation. Microsomes were prepared from the pooled livers of 2 to 4 mice as described by Kinoshita et al. (11). The microsomes were resuspended in 0.25 m sucrose:0.05 m Tris (pH 7.5) and immediately added to solutions of BaP and DNA. These incubation solutions contained 100 μg radiolabeled BaP in 0.2 ml acetone, 250 μmol Tris (pH 7.5), 0.5 μmol EDTA (pH 8.0), 10 mg calf thymus DNA, 50 μmol glucose-6-phosphate, 5 μmol NADPH, 10 units glucose-6-phosphate dehydrogenase, and 0.5 ml microsomal suspension in a total volume of 5 ml. The solutions were incubated at 37° for 2 hr with moderate shaking in brown bottles. The reactions were terminated by the addition of 5 ml of 2% lauryl sulfate; then the DNA was extracted as described above for epidermal DNA. The DNA was washed sequentially with 70% ethanol (4°), 100% ethanol (4°), 100% ethanol and acetone, and dried under a stream of nitrogen.

Enzyme Degradation and Column Chromatography of DNA. Purified DNA was enzymatically degraded to deoxyribonucleosides with DNase I, venom phosphodiesterase, and al-
kaline phosphatase as described by Baird and Brookes (1). The degraded DNA was chromatographed on a Sephadex LH-20 column (40 x 2.0 cm) equilibrated with 30% methanol as described by Baird and Brookes (1) with the modification that a 800-ml linear gradient was used. Samples of the enzymatically degraded DNA for HPLC were concentrated by lyophilization, dissolved in 40% methanol, filtered through a Millipore FHP0 013 000 filter (95% of the radioactivity was recovered), and separated on a Waters chromatograph equipped with a C18-@Bondapak column (3.9 mm x 30 cm). The column was eluted with a 10 to 100% methanol linear gradient for 30 min, at a flow rate of 2 ml/min.

**Analytical Measurement.** Radiolabeled samples were dissolved in 15 ml of ACS scintillation (Amersham/Searle Corp.) and counted in a scintillation counter Isocap/300 (Amersham/Searle Corp.). Counting efficiencies determined by external standard channel ratios were 27 to 30% for [3H]BaP and 69 to 72% for [14C]BaP. The amount of DNA was determined by the fluorometric procedure of Kissane and Robins (12), and the amount of protein was determined by the dye-binding assay of Bradford (3).

**RESULTS**

**Formation of BaP Adducts in Epidermal DNA.** BaP-conjugated DNA was obtained as described in ‘Materials and Methods’ from the epidermis of 15 mice treated with 25 μg [3H]BaP (100 μCi) at 7 hr prior to sacrifice. The DNA was enzymatically degraded to deoxyribonucleosides and chromatographed on a Sephadex LH-20 column. The elution profile of the radiolabeled products contained 3 peaks (Chart 1A). Peak III was eluted similarly to the major in vivo peak described previously by others to contain N7[10(7β,8α,9α) or 9β-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene]deoxyguanosines (2, 8, 10, 13, 14, 17, 22, 26). Two hydrophilic peaks, Peaks I and II, were present in the elution profile. When [3H]BaP-conjugated epidermal DNA was enzymatically degraded to deoxyribonucleosides and chromatographed on HPLC equipped with a C18-μBondapak column, 2 peaks of radioactivity were present in the elution profile (Chart 1B). The tritium-labeled hydrophobic peak isolated by HPLC was mixed with [14C]-labeled Peak III isolated from microsomal catalyzed conjugation of [14C]BaP to calf thymus DNA by Sephadex LH-20 column chromatography. Upon being rechromatographed on HPLC, the tritium of the HPLC hydrophobic peak cochromatographed with the 14C of Peak III from Sephadex LH-20. Therefore, Peak III of the HPLC and Sephadex LH-20 elution profiles probably contain the same BaP adduct to epidermal DNA.

It was important to determine whether Peaks I and II represent BaP adducts in DNA or result from tritium exchange onto the deoxyribonucleosides in DNA. We, therefore, determined the products formed by 3-methylcholanthrene-treated mouse liver microsomes catalyzed binding of a mixture containing [3H]BaP and [14C]BaP to calf thymus DNA. Radioactivity present in the enzymatically degraded DNA was eluted in 4 major peaks from the Sephadex LH-20 column (Chart 2). Peaks I, III, and IV contained both tritium and 14C indicating the presence of the BaP molecule. Peak II contained only tritium and by thin-layer chromatography the tritium present in Peak II migrated as the 4 deoxyribonucleosides (data not shown). Therefore, Peak II is an artifact of the use of tritium-labeled BaP resulting from tritium exchange; however, Peak I contained authentic BaP conjugate of DNA.

The possibility that Peak I was the result of incomplete hydrolysis of BaP-conjugated DNA was investigated. Peak I isolated from [14C]BaP-conjugated calf thymus DNA was subjected to the standard enzymatic hydrolysis. Upon rechromatography with Sephadex LH-20, the elution profile contained only Peak I, indicating the resistance of Peak I to enzymatic degradation.

**Rate of Formation and Removal of BaP Adducts in Epider-**
mal DNA. BaP-conjugated epidermal DNA was isolated from the pooled epidermis of 15 to 23 mice at various times after the application of 25 μg [3H]BaP (100 μCi) per mouse. The isolated DNA was degraded enzymatically to deoxyribonucleosides and chromatographed on Sephadex LH-20 or HPLC to quantitate Peaks I and III of the elution profile (Chart 3). The formation of Peaks I and III reached a maximum in 7 hr and remained constant for 49 hr. An interval of 24 hr after the topical application of BaP to the backs of mice occurs during the plateau of maximum adduct formation and was, therefore, chosen in the study of the dose dependency of adduct formation.

Dose Dependency of the Formation of BaP Adducts in Epidermal DNA. The formation of BaP adducts identified as Peaks I and III was investigated as a function of the amount of [3H]BaP applied. Various amounts ranging from 0.01 to 300 μg [3H]BaP per mouse were applied to the backs of 15 to 20 mice, and the conjugated epidermal DNA was isolated from the pooled epidermis 24 hr after treatment, degraded enzymatically, and chromatographed on either a Sephadex LH-20 column or a Waters C18-Bondapak. As shown in Chart 4, the amount of Peaks I and III was found to increase as a linear function of dose. The formation of Peak III was greater than that of Peak I throughout this range of doses, being 7.2 compared to 6.0 fmol/μg DNA or 20% greater at 25 μg/mouse.

**DISCUSSION**

We have determined in BaP-conjugated epidermal DNA the amount of 2 adducts separable by either Sephadex LH-20 column chromatography or C18-Bondapak reverse-phase HPLC. The BaP adduct to DNA eluted in the hydrophobic region of Sephadex LH-20 and HPLC elution profiles (Peak III) is probably the major in vivo BaP adduct identified by others as N²-(10β,8α, 9α, or 9β-trihydroxy7,8,9,10-tetrahydrobenzo-(α)pyreneyl) deoxyguanosine (8, 10, 13, 14, 16, 21, 23–25, 27). The second major peak (Peak I) in epidermal DNA was eluted in the hydrophilic region of the elution profile from Sephadex LH-20 and HPLC. We have confirmed that Peak I contained a 14C-radiolabeled adduct of BaP, resistant to further enzymatic degradation by DNase I, venom phosphodiesterase, and alkaline phosphatase (18–20).

The extent of formation of the 2 adducts of BaP to epidermal DNA reached a maximum in 7 hr and remained constant for the next 49 hr. The stability of the BaP adducts indicates that mouse epidermis does not excise BaP bound to DNA any more rapidly than do mouse embryo cells, which remove BaP adducts to deoxyguanosine with a half-time greater than 72 hr (23).

The formation of adducts of BaP to epidermal DNA was linear with dose throughout the dose range tested, which included doses several orders of magnitude below the lowest in which carcinogenicity testing is feasible in mouse skin. Linearity between BaP dose and adducts to DNA have been noted in rat liver at lower doses, but departures from linearity were found at higher doses in conjunction with induction of BaP-metabolizing enzymes (16). The explanation why the induction of BaP-metabolizing enzymes did not affect the linearity of adduct formation to epidermal DNA is not clear, although it is possible that enzyme induction occurred after most of the BaP was no longer present in the epidermal cells. The BaP persistence half-time was only about 2 to 3 hr, while enzyme induction generally requires 24 to 48 hr (21). Although the formation of BaP adducts to epidermal DNA was qualitatively similar throughout the dose range tested, it remains to be determined to what extent these adducts are an indicator of carcinogenic potency at low doses in which carcinogenicity testing is not feasible.

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