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INHIBITION OF EPIDERMAL METABOLISM AND DNA-BINDING OF BENZO[A]PYRENE BY ELLAGIC ACID

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Summary: Ellagic acid, a common plant phenol, was shown to be a potent inhibitor of epidermal microsomal aryl hydrocarbon hydroxylase (AHH) activity in vitro, and of benzo[a]pyrene (BP)-binding to both calf thymus DNA in vitro and to epidermal DNA in vivo. The in vitro addition of ellagic acid (0.25-2.0 μM) resulted in a dose-dependent inhibition of AHH activity in epidermal microsomes prepared from control or carcinogen-treated animals. The I_{50} of ellagic acid for epidermal AHH was 1.0 μM making it the most potent inhibitor of epidermal AHH yet identified. In vitro addition of ellagic acid to microsomal suspensions prepared from control or coal tar-treated animals resulted in 90% inhibition of BP-binding to calf thymus DNA. Application of ellagic acid to the skin (0.5-10.0 μmol/10 gm body wt) caused a dose-dependent inhibition of BP-binding to epidermal DNA. Our results suggest that phenolic compounds such as ellagic acid may prove useful in modulating the risk of cutaneous cancer from environmental chemicals.

The environmental oncogen BP is carcinogenic only after its metabolic transformation to a chemically reactive intermediate, which can then covalently bind to cellular macromolecules (1,2). There is general agreement that these covalent interactions with DNA are essential for BP carcinogenesis in skin (3,4). One of the isomers of BP-7,8-diol-9,10-epoxide is a known mutagen and is believed to be the ultimate carcinogenic metabolite of BP (5). Its production is catalyzed by the cytochrome P-450-dependent microsomal enzyme AHH and the non-P-450-dependent microsomal enzyme epoxide hydrolase (6), both of which are present in the skin (7,8). The importance of these enzymes in carcinogenesis has led to a continuing search for inhibitors which could be useful in modifying the cancer-causing effects of the PAHs.

Abbreviations used: BP, benzo[a]pyrene; AHH, aryl hydrocarbon hydroxylase; PAH, polycyclic aromatic hydrocarbon; 3-MC, 3-methylcholanthrene; DMSO, dimethyl sulfoxide; TKM-buffer, 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5.

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Several common plant phenols have recently been shown to inhibit the mutagenicity of a nitrosation mixture of nitrite and methylurea and PAHs in the Ames mutagen assay using S. typhimurium (9,10). We now report that ellagic acid, a phenolic product of the degradation of certain plant tannins, inhibits epidermal BP metabolism and the enzyme-mediated binding of BP to epidermal DNA.

MATERIALS AND METHODS

Chemicals: (G-^{3}H)-BP (specific activity 25 Ci/mmol) was purchased from Amersham Searle (Chicago, IL). Protease (Type XI), m-cresol, 8-hydroxyquinoline, 3-MC, NADPH, calf thymus DNA (Type I) and ribonuclease A (Type III-A) were purchased from Sigma Chemical Co. (St. Louis, MO). Standard coal tar solution (USP) was used. Ellagic acid dihydrate and > 99% pure phenol were purchased from Aldrich Chemical Co., (Milwaukee, WI). All solvents used were of HPLC grade and were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI).

Animals: Sperm-positive pregnant Sprague-Dawley rats of known insemination date were obtained from the Holtzman Rat Farm (Madison, WI). Neonatal rats born in situ were allowed to suckle until the 4th day after birth, withdrawn from their mothers, and used in the experiments. The advantages of using neonatal rodents for studies on cutaneous drug metabolism have been described previously (8).

Treatment of animals: The animals were treated with a single topical application of USP coal tar solution (1 ml/100 gm) or 3-MC (5 mg/100 gm in acetone) 24 hours prior to sacrifice. Controls received acetone alone. Coal tar which contains multiple PAHs, and 3-MC are good inducers of cutaneous carcinogen-metabolizing enzymes (11). Epidermal microsomes were prepared essentially as described earlier (7,8) and used for the in vitro DNA-binding and enzyme studies. For the in vivo DNA-binding studies animals were treated with a single topical application of coal tar or vehicle (DMSO). In experiments to assess the effect in vivo of ellagic acid the compound was topically applied to skin 1 hour prior to coal tar or DMSO application. All animals were then treated with 1 nmol ^{3}H-BP (in acetone) 2 hours prior to sacrifice. A 2 hour interval for metabolism of BP was found to be optimal (data not presented) and was chosen for these studies. The animals were killed by decapitation and the skins removed. Epidermis was separated from dermis as previously described (7,8). All subsequent operations were carried out at 0-4°C. The epidermis was then minced with scissors in 0.1 M phosphate buffer pH 7.4 containing 10 mM EDTA. The minced tissue was homogenized with a Polytron Tissue Homogenizer (Brinkmann Instruments, Switzerland) equipped with an ST-20 generator (8).

DNA extraction from epidermis: The DNA from minced epidermal homogenates was extracted essentially as described by Kates and Beeson (12), with an additional incubation step using protease K (0.5 mg/ml). A second extraction was performed using Kirby's phenol (13) before precipitation with cold 100% ethanol. The DNA was then digested with ribonuclease A (1000 units/ml), washed 3 times with acetone and dried under a stream of nitrogen. Extracted DNA was then dissolved in 5 ml of 0.1 M sodium chloride pH 7.0 and estimated by measuring its absorption at 260 nm. The purity of the DNA was assessed by the absorbance ratios A260/A280 $\geq$ 1.98 and A260/230 $\geq$ 2.21 (14). Aliquots were counted on a Packard TriCarb 460 CD liquid scintillation spectrometer to determine the amount of ^{3}H-BP bound to epidermal DNA.

In vitro DNA-binding experiments: The incubation system employed was similar to that described by Hesse et al (15). After incubation, the reaction mixtures

were centrifuged at 105,000 xg to isolate the microsomes, and digested with SDS to extract any traces of protein. The extraction procedure was similar to that described by Lesca et al (16). DNA estimation and binding to BP was then carried out as described above.

Enzyme assay: AHH activity in epidermal microsomes was determined by a modification of the method of Nebert and Gelboin (17), the details of which have been described earlier (7). The quantitation of phenolic BP metabolites was based on comparison to fluorescence of a 3-OH-BP standard. Protein was determined, after precipitation with trichloroacetic acid, by the procedure of Lowry et al using bovine serum albumin as a reference standard (18).

RESULTS

Effect of ellagic acid on epidermal microsomal enzyme-mediated binding of ^{3}H-BP to calf thymus DNA.

Epidermal microsomes prepared from animals treated with coal tar caused a 215% increase in binding of BP to calf thymus DNA as compared to microsomes prepared from control animals. The *in vitro* addition of ellagic acid, at a concentration of 0.5 mM to microsomal incubation systems from either control or coal tar-treated animals resulted in 90% inhibition of BP-binding to calf thymus DNA (Table 1).

Effect of ellagic acid on enzyme-mediated binding of ^{3}H-BP to epidermal DNA.

A single topical application of coal tar solution to neonatal rats caused a 3.4-fold increase over controls in BP-binding to DNA extracted from the epidermis (Table 2). One topical application of ellagic acid at the con-

Table 1: *In vitro* effect of ellagic acid on epidermal microsomal enzyme-mediated binding of ^{3}H-BP to calf thymus DNA.

Microsomal Preparations[1]	^{3}H-BP-bound to DNA (pmol/mg DNA)	Percent Inhibition
Control	3.40 ± 0.68[2]	--
Control + Ellagic Acid	0.32 ± 0.04	91
Coal Tar	10.70 ± 1.26	--
Coal Tar + Ellagic Acid	1.10 ± 0.32	90

[1] Incubation mixtures consisted of 20 mg of epidermal microsomal protein from control or coal tar pretreated animals, 12 mg of calf thymus DNA, 10 nmol ^{3}H-BP and 8.8 mg NADPH in 10 ml of TKM-buffer. In incubations with ellagic acid, the reaction mixture contained 5 mol in 0.67 ml of DMSO. The addition of DMSO at this concentration had no effect on DNA-binding.

[2] Mean + SEM of four experiments.

Table 2: Effect of topical application of ellagic acid on the enzyme-mediated binding of ^{3}H-BP to epidermal DNA.

Treatments[1]	^{3}H-BP-bound to DNA (pmol/mg DNA)	Percent Inhibition
Control	2.51 ± 0.37[2]	--
Control + Ellagic Acid	0.34 ± 0.07	86
Coal Tar	8.52 ± 1.01	--
Coal Tar + Ellagic Acid	0.60 ± 0.16	93

[1]The animals received a single topical application of coal tar or acetone 24 hours prior to sacrifice. In animals where the effect of ellagic acid was studied, the phenol was applied to the skin 1 hour prior to coal tar or acetone-treatment. All animals then received a single topical treatment of ^{3}H-BP (1 nmol in 100 l acetone) 2 hours prior to sacrifice. For each determination the skin from 10 animals was pooled.

[2]Mean ± SEM of four experiments.

centration 5 µmol/10 gm body wt to control or to coal tar-treated animals caused marked decreases in the binding of BP to epidermal DNA (Table 2).

Topical application of ellagic acid at concentrations ranging from 0.5-10 µmol/10 gm body wt resulted in a dose-dependent inhibition of enzyme-mediated binding of ^{3}H-BP to epidermal DNA (Fig. 1). Sixty per cent inhibition was observed at the lowest concentration tested (0.5 µmol/animal) and > 95% inhibition occurred at a dose of 10 µM (Fig. 1).

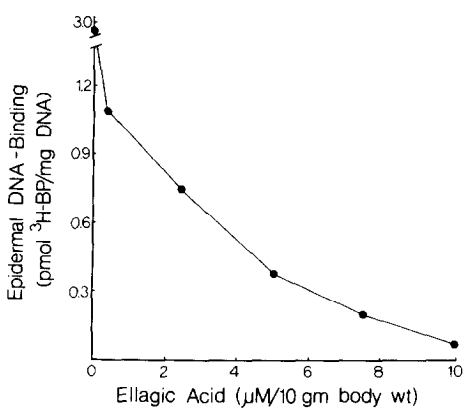

Fig. 1 Dose-dependent inhibition of enzyme-mediated binding of ^{3}H-BP to epidermal DNA by ellagic acid. Varying concentrations of ellagic acid were applied to the skin of the animals as described in Methods.

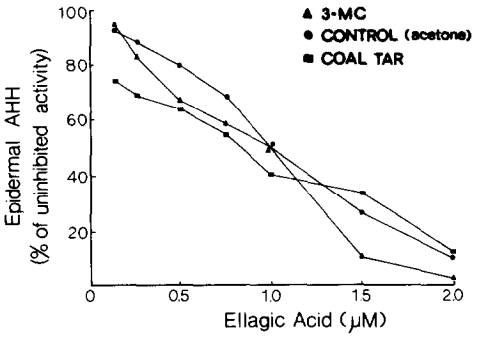

Fig. 2 Dose-dependent inhibition of epidermal AHH activity by ellagic acid. Varying concentrations of ellagic acid were added in 50 μl of DMSO to incubation mixtures containing epidermal microsomes prepared from control, 3-MC or coal tar-treated animals.

Effect of ellagic acid on epidermal microsomal AHH activity.

In further experiments, the effect of ellagic acid on the rate of epidermal BP metabolism was assessed. The in vitro addition of ellagic acid at concentrations ranging from 0.2-2.0 μM resulted in a dose-dependent inhibition of AHH activity in epidermal microsomes prepared from control, 3-MC or coal tar-treated animals (Fig. 2). At a concentration of 2 μM, inhibition of AHH activity was > 85%. The I_{50} values for several inhibitors of epidermal AHH activity are provided in Table 3 and, as can be seen, ellagic acid is the most potent inhibitor of epidermal AHH activity yet studied.

Table 3: I_{50} VALUES FOR MONOOXYGENASE INHIBITORS OF AHH ACTIVITY IN EPIDERMAL MICROSOMES.

INHIBITOR	I_{50} for AHH	
	CONTROL MICROSOMES	3-MC-INDUCED MICROSOMES
ELLAGIC ACID	1.0×10^{-6}M	1.0×10^{-6}M
α-NAPHTHOFLAVONE	5.2×10^{-5}M	1.3×10^{-6}M
METYRAPONE	3.2×10^{-5}M	6.8×10^{-6}M
SKF-525A	1.2×10^{-5}M	1.0×10^{-6}M
1-BENZYLIMIDAZOLE	5.6×10^{-5}M	2.2×10^{-5}M

I_{50} = Concentration of inhibitor which decreased catalytic activity by 50%.

DISCUSSION

The data presented here indicate that ellagic acid largely inhibited the epidermal microsomal enzyme-mediated binding of BP to calf thymus and to epidermal DNA. In microsomes prepared from animals pretreated with coal tar solution (USP) there was a 3.4-fold increase in the binding of BP to calf thymus DNA. The addition of ellagic acid to this incubation system caused substantial inhibition of the binding reaction and it is important to emphasize that this inhibition occurred in microsomal preparations from both control and coal tar-treated animals.

The effect of *in vivo* application of ellagic acid on the enzyme-mediated binding of BP to epidermal DNA resulted in marked decreases in binding of BP to epidermal DNA. This inhibition was observed in both untreated and coal tar-treated animals. Topical application of different concentrations of ellagic acid caused a dose-dependent inhibition of BP-binding to epidermal DNA.

Ellagic acid was also capable of inhibiting the epidermal metabolism of BP. Increasing concentrations of ellagic acid added to incubation mixtures containing either control or carcinogen-induced epidermal microsomes caused a dose-dependent inhibition of epidermal AHH activity. As compared to several other monooxygenase inhibitors, ellagic acid was found to be the most potent yet studied in this regard.

Recent studies have shown that ellagic acid inhibits the mutagenicity of BP in bacteria and in animal cells in culture (10). In these studies it was postulated that the inhibitory effect of ellagic acid may be due to its interaction and subsequent incorporation into BP-diol-epoxides. This inactivation of the diol-epoxide would reduce its capability to undergo covalent binding to DNA (19). Our data indicate that ellagic acid is a potent inhibitor of the cytochrome P-450-dependent monooxygenase system in the skin and that inhibition of the metabolic transformation of the PAH, BP by microsomal enzymes results in its reduced binding to DNA.

The potency of this plant phenol in inhibiting carcinogen metabolism suggests that further study of this class of compounds on PAH carcinogenesis in the skin is clearly warranted.

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