Metabolic Activation of Benzo(a)pyrene and Binding to DNA in Cultured Human Bronchus

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SUMMARY

Human bronchus is one target site for the carcinogenic action of tobacco smoke, which contains chemical carcinogens, including benzo(a)pyrene. Human bronchi were obtained from surgery or "immediate" autopsy and then cultured in a chemically defined medium. The cultured bronchi were exposed to either benzo(a)pyrene or its metabolites, and their levels of binding to DNA were measured. One of the benzo(a)pyrene metabolites, (—)-trans-7,8-diol, is more active in binding to DNA than benzo(a)pyrene and several of its metabolites, including (—)-trans-4,5-diol, (—)-trans-9,10-diol, and phenols. The predominant metabolite formed by human bronchus from the (—)-trans-7,8-diol is found by high-pressure liquid chromatographic analysis to be the diol-epoxide r-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydronobenzo(a)pyrene. The results suggest that this diol-epoxide is the major benzo(a)pyrene metabolite bound to DNA in human bronchus.

INTRODUCTION

BP is a chemical carcinogen contaminating the environment; approximately 1300 tons of BP are emitted into the air of the United States per year (25). BP is also a major carcinogen found in tobacco smoke, the inhalation of which is associated with an increased risk of bronchogenic carcinoma (30). Model systems for studying carcinogenesis in human target tissues are being developed to link investigations using experimental animals with human cancer (8). As part of these investigations, cultured human bronchus has been shown to metabolize BP to intermediates that bind to cellular macromolecules, including DNA (11, 12). A 75-fold interindividual variation was found in the binding levels of BP. Binding of BP to DNA was also inhibited by 7,8-benzoflavone and to a lesser extent by vitamin E and by disulfiram (10). BP is converted by the microsomal mixed-function oxidases, epoxide hydratase, and various conjugates to at least 20 to 25 metabolites. These include 5 phenols, 3 quinones, 3 dihydrodilols, several epoxides, and glutathione and glucuronide conjugates of many of the hydroxylated metabolites (3, 13, 14, 16, 22, 23, 26–28, 34, 36–38). Recently, we reported that the 3 trans-dihydroidiolos formed metabolically from BP with rat liver microsomes are all optically active (34). Further studies indicated that they are all optically pure (—) enantiomers (36).

Gelboin (6) reported that microsomes catalyzed the binding of BP to DNA. Subsequently, Borgen et al. (3) showed that the 7,8-diol of BP bound more readily to DNA than BP in the presence of microsomes and NADPH. Sims et al. (29) showed that a synthetic 7,8-diol-9,10-epoxide of BP was chemically very reactive in binding to DNA and suggested that a diol-epoxide may be the reactive intermediate. A synthetic racemic diol-epoxide r-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide I) is the most mutagenic of all known BP derivatives in mammalian cells (14). The stereoisomeric diol-epoxide r-7,8-dihydroxy-c-9,10-oxo-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide II) also was mutagenic in some strains of Salmonella typhimurium (19) and cultured Chinese hamster V79 cells (14, 24, 32).

However, diol-epoxide I was more mutagenic in cultured Chinese hamster V79 cells than diol-epoxide II was (14, 24). We have also reported (34, 37) that a single enantiomer of diol-epoxide I is predominantly formed from the (—)-r-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene ([—]-trans-7,8-diol) by rat liver microsomal mixed-function oxidases and epoxide hydratase. The precursor of the enzymatically formed diol-epoxide I, trans-7,8-diol, is an optically pure (—) enantiomer (36, 37) and, in the presence of mixed-function oxidase activity, binds covalently to DNA in vitro and to DNA

1 The abbreviations used are: BP, benzo(a)pyrene; HPLC, high-pressure liquid chromatography; (7,10/8,9)-tetro, tetrahydroxytetrahydrobenzo(a)pyrene in which the 10-OH is cis and 8-OH and 9-OH are trans to the reference 7-OH, respectively (other tetros and triols are similarly designated).

Received October 4, 1976; accepted January 14, 1977.
and RNA in cultured mammalian cells (3, 4, 21, 24, 29, 31). The racemic trans-7,8-diol was more mutagenic than any known BP metabolite in cultured mammalian cells when it is further metabolized (14). The racemic 7,8-epoxide in which one of the enantiomers is the specific precursor of (−)-trans-7,8-diol (36) is carcinogenic on mouse skin (17). The BP metabolite that binds to cellular DNA in cultured hamster embryo cells (4, 29) and in BP-treated human bronchi (8) has been suggested to be a non-K-region diol-epoxide. An adduct formed between the C_{1,6} position of diol-epoxide I and the 2-amino group of guanine is the major adduct found in RNA obtained in vitro from cultured bovine bronchus incubated with BP (31). The above findings suggest that the enzymatically formed diol-epoxide I (14, 37) may be the predominant, ultimate carcinogenic form of BP. The results reported here further delineate the activation of BP by the human bronchial mucosa.

MATERIALS AND METHODS

[3H]BP (generally labeled; specific activity, 5 Ci/mmole) was purchased from Amersham/Searle Corp., Arlington Heights, Ill., and purified by a gravity-flow silica gel column (38). Liver microsomes were prepared from male Sprague-Dawley rats treated with 3-methylcholanthrene as previously described (16). Protein content was determined by the method of Lowry et al. (18) with RNase A as the protein standard. BP metabolites were obtained by incubating [3H]BP (50 mCi) in a 200-ml reaction mixture containing Tris-HCl (10 mmoles), liver microsomes (240 mg of protein), MgCl₂ (0.6 mmole), and NADPH (200 mg), pH 7.5. The reaction mixture was incubated at 37°C for 1 hr. BP and its metabolites were extracted from the reaction mixture twice with acetone (200 ml) and ethyl acetate (400 ml), and the resulting organic phase was dehydrated with magnesium sulfate and evaporated to dryness under reduced pressure. BP metabolites were dissolved in benzene (1 ml) and loaded onto a 0.75- x 25-cm column containing silicic acid (Bio-Sil A, 100 to 200 mesh; Bio-Rad, Richmond, Calif.) previously equilibrated with benzene. The column was first eluted with benzene. At the end of the 10th fraction (1.4 ml/fraction), the column was eluted with a linear gradient of 0 to 50% methanol in benzene with 130 ml in each of the solvent reservoirs. The metabolites were completely eluted at the end of the 70th fraction. The elution profile (see Chart 1) was determined by liquid scintillation counting. Radioactive BP purchased from commercial sources was routinely purified by this column chromatography with benzene as the eluent.

Purification of BP Diols by HPLC. The 3rd peak eluted from the silica gel column (see Chart 1) contains 3 BP diols and 3 quinones. The diols were purified by HPLC with a DuPont 6.2-mm (inside diameter) x 25-cm Zorbax octadecyltrimethylsilane column on a Spectra-Physics Model 3500 liquid chromatograph. The column was eluted at room temperature with a linear gradient of 65% methanol in water to 100% methanol for 50 min. The solvent flow rate was 0.8 ml/min. Under these conditions, the retention times of 9,10-diol, 4,5-diol, 7,8-diol, 9-OH-BP, 3-OH-BP, 1,6-quinone, and 3,6-quinone were 23.0, 37.5, 42.8, 57.7, 61.3, 63.6, and 65.1 min, respectively. The purities of the separated diols were assayed by rechromatography on HPLC and by liquid scintillation counting of the collected HPLC fractions.

Cultured Human Bronchus. Human bronchial specimens were obtained at surgery from 2 different male patients with squamous cell carcinoma of the lung (ages 56 and 47) and from 1 female patient with adenocarcinoma of the lung (age 54). The patients had not received previous cancer therapy. Bronchial specimens were also obtained at "immediate" autopsy from a male patient (age 42) who died of head trauma. Explants (1 x 1 cm) of grossly normal-appearing bronchi from each patient were cultured in a chemically defined medium (CMRL-1066; Grand Island Biological Co., Grand Island, N. Y.) containing hydrocortisone hemisuccinate (0.1 µg/ml; The Upjohn Co., Kalamazoo, Mich.), crystalline bovine insulin (1 µg/ml; Eli Lilly & Co., Indianapolis, Ind.), β-retinyl acetate (0.1 µg/ml; Hoffmann-La Roche Inc., Nutley, N. J.), penicillin G (100 units/ml), and streptomycin (100 µg/ml; Grand Island Biological Co.) on a rocker platform in an atmosphere of 50% O₂, 45% N₂, and 5% CO₂ as previously described (1). To allow the levels of aryl hydrocarbon hydroxylase induced by exogenous agents to reach basal levels, the culture was maintained for 7 days before treatment with either BP or its metabolites.

DNA Binding in Cultured Human Bronchus. After 7 days, the cultured human bronchus was treated with either [3H]BP (8 nmoles/ml; 5 Ci/mmole) or the metabolites of [3H]BP (8 nmoles/ml), which were dissolved in dimethyl sulfoxide (final concentration, 0.5%) and added to the culture medium for 24 hr. The bronchial mucosa was then scraped from the supporting connective tissue of 5 explants/experimental variable. The highly purified DNA was isolated from the cells on CsCl gradients, and the radioactivity bound to DNA was determined as described previously (11, 12). Previous studies with the use of similar conditions and the substitution of [14C]BP for [3H]BP have demonstrated that the bound radioactivity was not due to tritium exchange and that the intraidividual variation due to experimental methodology was minimal (coefficient of variation, 0.1) (11). One explant/experimental variable was fixed in 3% gluteraldehyde and was examined by high-resolution light microscopy for monitoring cell viability and autoradiography (11).

Metabolism of (−)-trans-7,8-Diol in Cultured Human Bronchus. Five explants (1 x 1 cm) of grossly normal-appearing bronchus from a female patient with adenocarcinoma of the lung and a patient without cancer who died due to head trauma were cultured for 7 days as described above and treated with [3H]-(−)-trans-7,8-diol (12.8 nmoles; specific activity, 322 mCi/mmole) for 24 hr. The media and the supernatant were pooled and added to the culture medium for 24 hr. The bronchial mucosa was then scraped from the supporting connective tissue of 5 explants/experimental variable. The highly purified DNA was isolated from the cells on CsCl gradients, and the radioactivity bound to DNA was determined as described previously (11, 12). Previous studies with the use of similar conditions and the substitution of [14C]BP for [3H]BP have demonstrated that the bound radioactivity was not due to tritium exchange and that the intraidividual variation due to experimental methodology was minimal (coefficient of variation, 0.1) (11). One explant/experimental variable was fixed in 3% gluteraldehyde and was examined by high-resolution light microscopy for monitoring cell viability and autoradiography (11).

Analysis of (−)-trans-7,8-Diol Metabolism by HPLC. A Spectra-Physics Model 3500 liquid chromatograph fitted with a DuPont 6.2-mm inside diameter x 25-cm Zorbax octadecyltrimethylsilane column was used. The column was eluted at room temperature with a linear gradient of 65% methanol in water to 100% methanol for 50 min. The solvent flow rate was 0.8 ml/min. Under these conditions, the retention times of 9,10-diol, 4,5-diol, 7,8-diol, 9-OH-BP, 3-OH-BP, 1,6-quinone, and 3,6-quinone were 23.0, 37.5, 42.8, 57.7, 61.3, 63.6, and 65.1 min, respectively. The purities of the separated diols were assayed by rechromatography on HPLC and by liquid scintillation counting of the collected HPLC fractions.
was eluted at room temperature with a linear gradient of 60% methanol in water to 100% methanol for 100 min. The solvent flow rate was 0.8 ml/min. The (−)-trans-7,8-diol and its metabolites obtained as described above were cochromatographed with 14C-labeled BP tetrols and triol prepared by treating BP [7-14C]diol-epoxide I (20) with water in the presence of NADPH (33, 37).

RESULTS AND DISCUSSION

Preparation of [14H]BP Metabolites. The [14H]BP metabolites obtained by incubating [14H]BP with rat liver microsomes were separated by a gravity flow silica gel column into 4 peaks (Chart 1). HPLC analysis (27, 38) indicated that the 4 chromatographic peaks in Chart 1 contain BP, BP phenols, BP diols and quinones, and a complex mixture of more polar BP metabolites, respectively. The BP phenol mixture contains predominantly 3-phenol. The 3 dihydrodiols were isolated by HPLC from Peak C. These 3 dihydrodiols have recently been found to be optically pure (−)-trans-diols (36, 37). Under conditions described in Chart 2, Peak D is separated into more than 10 peaks, of which only 1 has been identified to date as a BP tetrol (S. K. Yang, D. W. McCourt, and H. V. Gelboin, unpublished results). As shown below and also reported previously, the formation of tetrols from BP is an indication that a diol-epoxide is formed from BP (14, 37).

The finding that the 1st peak in Chart 1 contains only BP has been routinely used in our laboratory to purify the BP purchased from commercial sources. This procedure is simple, safe, and effective and is highly recommended for the purification of radioactive polycyclic aromatic hydrocarbons.

Binding to Human Bronchial Mucosal DNA. Table 1 shows that (−)-trans-7,8-diol binds to human bronchial mucosal DNA 5- to 23-fold higher than BP and 2.5- to 8-fold higher than (−)-trans-9,10-diol. The binding levels of a mixture of BP phenols and (−)-trans-4,5-diol are lower than the...

![Chart 2](image-url)
parent hydrocarbon, BP. The higher DNA binding activity of
the enzymatically formed trans-7,8-diol was also found in vitro in the presence of rat liver microsomal enzymes (3).

In the absence of microsomal mixed-function oxidases, trans-7,8-diol does not bind to DNA (3) nor is it mutagenic in cultured Chinese hamster V79 cells (14). We therefore investigated the possible chemical identity of the DNA-binding metabolite derived from (−)-trans-7,8-diol in cultured human bronchial cells. The trans-7,8-diol constitutes 3 to 6% of the total BP metabolites when human bronchi are treated with BP.3

Diol-epoxide I Is the Predominant DNA-binding BP Metabolite. Chart 2 shows that diol-epoxides are formed from (−)-trans-7,8-diol in 2 different human bronchial explants. BP diol-epoxides I and II are unstable in aqueous media and cannot be detected directly (14, 37). The enzymatic formation of diol-epoxides is indicated by the detection of their hydrolysis products and NADPH reduction products (14, 33, 37). Under the HPLC conditions as similarly described in Chart 2, the hydrolysis and NADPH reduction products of diol-epoxide II are separated from those of diol-epoxide I (34, 37). Chart 2 indicates that diol-epoxide I is a major metabolite formed from (−)-trans-7,8-diol by the human bronchial mucosa. A small but detectable amount of products (tetrols) (37) that are derived from diol-epoxide II (retention time, 24.5 and 34.0 min in Chart 2A) are also formed.

Fig. 1. Human bronchial explants were cultured with either BP (A) or (−)-trans-7,8-diol (B) under conditions described in "Materials and Methods." Autoradiograms were prepared as previously described in detail (2). One-μm sections. Toluidine blue, × 880.
The relative amounts of diol-epoxides I and II actually formed by human bronchial mucosa are difficult to assess due to their reactivity in binding to cellular macromolecules (29, 31). However, diol-epoxide II was the minor metabolite (ca. 10% of diol-epoxide I) formed from the (−)-trans-7,8-diol in hepatic microsomes (37).

Further Evidence That the Major Human Bronchial Mucosal DNA-binding BP Metabolite is Derived from (−)-trans-7,8-Diol. Cellular localization of BP bound to macromolecules, primarily protein, can be determined by an autoradiographic assay (2). Bronchial epithelial cells bind approximately 4-fold more BP than stromal fibroblasts (11). Incubation with (−)-trans-7,8-diol leads to a greater level of binding to bronchial epithelial cells than with the parent compound, BP (Fig. 1). In both cases, binding was found in both nuclear and cytoplasmic sites of mucous, ciliated, and basal cells. Since diol-epoxide I is the major metabolite derived from the (−)-trans-7,8-diol (Chart 2), the major DNA-binding metabolite (Fig. 1) is therefore the diol-epoxide I, which is enzymatically derived from (−)-trans-7,8-diol. The nature of the adduct formed between the metabolite of BP and DNA, however, also suggests that diol-epoxide I is the major metabolite formed (31). While the adduct between the intermediate(s) of BP and DNA has not as yet been isolated and directly identified, the above results indicate that the predominant metabolite of BP bound to DNA is a single enantiomer of diol-epoxide I, which is enzymatically derived from the optically pure (−)-trans-7,8-diol. The (−)-trans-7,8-diol derived from BP by rat liver microsomal mixed-function oxidases and epoxide hydrolase has been previously shown to an optically pure (−) enantiomer (36, 37). This (−)-trans-7,8-diol is also further metabolized to a single enantiomer of diol-epoxide I by the rat liver microsomal mixed-function oxidases and epoxide hydrolase has been previously shown to an optically pure (−) enantiomer (36, 37). This (−)-trans-7,8-diol is also further metabolized to a single enantiomer of diol-epoxide I by the rat liver microsomal mixed-function oxidases and epoxide hydrolase has been previously shown to an optically pure (−) enantiomer (36, 37). This (−)-trans-7,8-diol is also further metabolized to a single enantiomer of diol-epoxide I by the rat liver microsomal mixed-function oxidases and epoxide hydrolase has been previously shown to an optically pure (−) enantiomer (36, 37). This (−)-trans-7,8-diol is also further metabolized to a single enantiomer of diol-epoxide I by the rat liver microsomal mixed-function oxidases and epoxide hydrolase has been previously shown to an optically pure (−) enantiomer (36, 37).

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ADDITIONAL INFORMATION

The HPLC-isolated [3H]7,8-diol, which was formed metabolically from [3H]BP, was mixed with nonradioactive (+)-trans-7,8-diol and the mixture was reacted with (−)menthoxyacetyl chloride. The resulting dimenthoxyacetate of the (−)-trans-7,8-diol has an optically pure (−) trans-7,8-diol enantiomer.


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_Cancer Res_ 1977;37:1210-1215.

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