Mutagenesis and DNA Binding of Benzo(a)pyrene in Cocultures of Rat Hepatocytes and Human Fibroblasts

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ABSTRACT

The genotoxicity of benzo(a)pyrene (BP) was investigated in combined cultures of rat hepatocytes and human diploid fibroblasts. Freshly isolated rat hepatocytes were shown to activate BP to a species which bound to and damaged hepatocyte and fibroblast DNA. A significant increase in the hypoxanthine-guanine phosphoribosyltransferase mutation frequency was induced when 10 to 100 μM BP was added to the cocultures. A comparative analysis of the binding of BP metabolites to hepatocyte and fibroblast DNA revealed that approximately 4 times more [³H]BP metabolites were bound to the fibroblast DNA than were bound to the hepatocyte DNA (per μg DNA). Activation of BP by the fibroblasts themselves was shown not to be the cause of the relatively greater binding of BP to fibroblast DNA than to the hepatocyte DNA. These results suggest that proximate and/or ultimately carcinogenic metabolites of BP are readily released from isolated hepatocytes and that the metabolites are sufficiently stable and long lived so as to bind to the DNA of an adjacent cell. The relative protection of the hepatocytic DNA from BP metabolites that generated in the cytoplasm of the hepatocyte may be significant in view of the observations that the liver is not under normal conditions a target of BP carcinogenicity in vivo.

INTRODUCTION

Polycyclic aromatic hydrocarbons such as BP are mutagenic, carcinogenic, and cytotoxic after metabolic activation by microsomal mixed-function oxygenases (20). The products of BP metabolism include organic solvent-soluble metabolites such as epoxides, BP phenols and their sulfate ester conjugates, dihydrodiols, quinones, diol-epoxides, triols, tetrahydro- and water-soluble glucuronide and glutathione conjugates (9, 18, 20, 45). The primary oxidation products may be reduced back to the parent hydrocarbon (48), may spontaneously isomerize to phenols, may be hydrated stereospecifically to their corresponding trans-diols (20, 45), or can be conjugated by glutathione-S-transferase (9, 10, 11, 18, 20, 45). Phenols and diols can be conjugated with sulfate (9) or glucuronide (18, 45) or can be further oxidized to products which are strongly electrophilic and can covalently bind to cellular macromolecules including DNA (2, 6, 14, 22, 45, 52). The major DNA adducts of BP found in vivo or in vitro resulted or can be conjugated by glutathione-S-transferase (9, 10, 18, 22, 43). Since the critical balances of activation and detoxification are disrupted in microsomal activation systems, it is valuable to study metabolism and DNA binding of carcinogens in intact cells or with cell-mediated assays (3, 11, 21, 23, 27-30, 35, 37, 38).

We (27, 33, 47) have recently developed methods to study some critical steps involved in carcinogenesis such as binding of carcinogen metabolites to DNA, DNA damage and repair, and mutagenesis in combined cultures of human diploid fibroblasts and freshly isolated rat hepatocytes. These culture systems are useful for in vitro studies of a wide variety of carcinogens because of the extensive metabolic capabilities of the hepatocytes. Since the human fibroblasts have little or no ability to activate carcinogens themselves, these cocultures offer a unique system to study the transfer of carcinogen metabolites from their point of activation, the hepatocytes, to adjacent cells in the culture. In this report, we present data to indicate that BP is activated by rat hepatocytes to metabolites, which pass out of the hepatocytes and are sufficiently stable and long-lived to ultimately induce DNA damage and mutations in human fibroblasts cultured with the hepatocytes.

MATERIALS AND METHODS

Mutagenesis Experiments. Human male diploid fibroblasts (GM 3468 Human Genetic Cell Repository) were maintained at passages 7 to 10 in MEM supplemented with amino acids and 10⁻⁶ m thymidine-10⁻⁶ m amethopterin-10⁻⁶ m hypoxanthine as reported previously (33). Mutagenesis experiments and the determination of the relative plating efficiency were performed as described earlier (33). Briefly, 24 hr prior to the addition of hepatocytes, 1.5 × 10⁶ fibroblasts were seeded per 60-mm-diameter tissue culture dish (Falcon Plastics, Oxnard, Calif.). During the subsequent 24-hr period, the fibroblasts attach and spread out to form a continuous monolayer with minimal multilayer formation.

Liver Cell Culture. Hepatocytes were isolated by perfusion of the

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³ The abbreviations used are: BP, benzo(a)pyrene; 9-hydroxy-BP, 9-hydroxybenzo(a)pyrene; MEM, minimal essential medium (Eagle's) with Earle's salts; HPRT; hypoxanthine-guanine phosphoribosyltransferase; BrdUrd, 5-bromo-2-deoxyuridine.
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livers of male Sprague Dawley rats (Charles Rivers Laboratories, Wilmington, Del.) with collagenase (33). Approximately 3.5 x 10^6 hepatocytes were plated over the fibroblast monolayers in MEM supplemented as above except that 5% fetal bovine serum (Sterile Systems Inc., Logan, Utah) was included to aid in the attachment of the hepatocytes to the fibroblasts. Cultures were left undisturbed for 4 hr, and then the medium was changed to serum-free MEM. The appropriate amount of BP (Aldrich Chemical Co., Milwaukee, Wis.; 1% dimethyl sulfoxide final concentration in the media in all experiments) was added to the combined cultures or cultures of fibroblasts alone at this time, and the cultures were incubated at 37° for 45 hr in the dark. After the carcinogen exposure, hepatocyte-fibroblast cocultures or cultures of fibroblasts alone were trypsinized, and the relative plating efficiency and the induced HPRT\(^{-}\) frequency were determined as described (33). Expression times of 4 to 6 days resulted in an optimal HPRT\(^{-}\) mutation frequency as was reported previously with cyclophosphamide (33).

**Determination of the Binding of BP to DNA.** Binding of \(^{3}H\)BP to hepatocyte and fibroblast DNA was determined as described previously (47). Seven to 10 days prior to being overlaid with hepatocytes, fibroblast cultures were trypsinized and diluted with enough fresh media (+20% fetal bovine serum) to seed 9 new culture dishes. BrdUrd (10 µM) was added to the media to substitute for thymidine in the fibroblast DNA. BrdUrd-substituted fibroblasts were allowed to grow to confluence in total darkness. When the fibroblast cultures were confluent, hepatocytes were added under reduced light (Westinghouse 4T12 high-output gold) as indicated above. After the media change, \(^{3}H\)BP (52 mCi/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) was added to the media (BrdUrd and serum-free) either directly or with an appropriate amount of unlabeled BP. Hepatocyte-fibroblast cocultures or cultures of fibroblasts alone were incubated with the \(^{3}H\)BP for 45 hr in the dark. The cultures were harvested, and the hepatocyte DNA was separated from the heavier fibroblast DNA as described earlier (47) except that an additional cesium chloride isolation step with the TV-865 vertical rotor (Dupont-Sorvall, Wilmington, Del.) was added to ensure complete separation of the normal density DNA from the hybrid-density DNA (BrdUrd substituted), and the specific activity of the DNA was determined (47).

**Determination of Single-Strand Breaks in Hepatocyte and Fibroblast DNA.** The average molecular weight of hepatocyte DNA and fibroblast DNA was determined by sedimentation on 5 to 20% alkaline sucrose gradients (46) in a SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 2.5 hr at 55,000 rpm. Hepatocyte DNA was labeled in vivo by giving rats injections of 100 µCi of \(^{3}H\)thymidine at 17, 20, and 24 hr after a two-thirds partial hepatectomy. Rats were allowed to recover from the surgery for at least 2 weeks. When recovery was complete, the livers were perfused and the \(^{3}H\)-labeled hepatocytes were plated over confluent monolayers of fibroblasts labeled previously with \(^{14}C\)thymidine (0.2 µCi/ml; 5 days; ICN Chemical Radioisotopes Div., Irvine Calif.) and were exposed to BP for 24 hr.

**BP Solubility Experiments.** Since BP is highly lipophilic, we investigated the solubility of BP in the media under the conditions of exposure used in the mutagenesis and DNA-binding experiments. Conditioned media were collected from cocultures of hepatocytes and fibroblasts, cultures of fibroblasts alone, hepatocytes alone, and from culture dishes with no cells after a 45-hr incubation period. A mixture of the proper concentration of unlabeled BP and \(^{3}H\)BP (0.1 µCi/ml) was added to the conditioned medium, and the mixture was stirred slowly on a magnetic stirrer at 37° for 24 hr. At the appropriate time, the media were centrifuged at 100,000 x g for 1 hr to sediment any BP that was not in solution. An aliquot of the supernatant was taken, and the solubilized BP was determined by liquid scintillation counting.

**RESULTS**

Data presented in Chart 1 indicate that BP activated by adult rat hepatocytes induced a significant increase in the frequency of HPRT\(^{-}\) mutant human fibroblasts (Chart 1B) at concentrations of BP which were not excessively toxic to the fibroblasts (Chart 1A). There was no significant increase in the HPRT\(^{-}\)mutagenic frequency in fibroblasts exposed to BP in the absence of hepatocytes (Chart 1, solid circles) suggesting that the fibroblasts themselves cannot activate BP to a mutagenic species but must rely on metabolites passed to them from the hepatocytes for the induction of mutations.

We had observed previously that even though hepatocytes were mainly responsible for the activation of BP in cocultures of hepatocytes and human fibroblasts, approximately 3 times more \(^{3}H\)BP was bound to the fibroblast DNA, and in separate experiments more unscheduled DNA synthesis was induced in the fibroblasts than in the hepatocytes (47). We have extended those previous observations of the binding of BP to hepatocyte and fibroblast DNA to a wider range of concentrations of BP. The results of those experiments (Chart 2) are consistent with our previous observations in that, at every concentration of BP examined, approximately 4-fold more \(^{3}H\)BP was bound to the fibroblast DNA than was bound to the hepatocyte DNA. We have shown previously that the substitution of the fibroblast DNA with BrdUrd does not enhance the binding of BP to the DNA (47). Also, activation of BP by the fibroblasts was not responsible for the greater binding of BP to the fibroblast DNA. Binding of \(^{3}H\)BP to the DNA of (BrdUrd-substituted) fibroblasts exposed to the BP in the absence of hepatocytes was 18, 6, 4, and 14% of the value shown in Chart 2 for the binding of BP to the fibroblast DNA in the cocultures (0.1 to 100 µM BP, respectively).

Although binding to hepatocyte and fibroblast DNA appeared to be linear from 0.1 to 100 µM BP, significant increases in the

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**Chart 1.** Cytotoxicity and mutagenicity of BP. Human fibroblasts were exposed to the indicated concentrations of BP in the presence (C) or the absence (F) of hepatocytes. Top, replating efficiencies of fibroblasts exposed to the indicated concentrations of BP. The ordinate is in log scale, and the results are expressed as a percentage of the control-plating efficiency. Bottom, frequency of HPRT\(^{-}\) mutant human fibroblasts expressed as number of mutants per 10^5 clonogenic survivors. The control background mutation frequency was 0.34 ± 0.18 x 10^-5 in 5 experiments. Points, the mean from 3 experiments. Bars, S.E. The induced mutation frequencies for fibroblasts exposed to BP in the presence of hepatocytes were significantly greater than control (p < 0.05) as determined by Student’s t test.
mutation frequency occurred only at and above 10 μM BP. One can determine from the binding data for 10, 50, and 75 μM BP that the average amount of carcinogen bound to the fibroblast can be determined from the binding data for 10, 50, and 75 μM BP. The magnitude of the decrease was approximately linear until 100 μM BP, the induced mutation frequency dropped sharply at 100 μM BP. This inconsistency among DNA binding, cellular toxicity, and the induced mutation frequency may be due to the relative increase in the proportion of phenol-oxide metabolites to diol-epoxide metabolites which was reported to occur at 100 μM BP (45). The presence of dideoxythymidine did not result in the accumulation of single-strand breaks in incubations up to 48 hr, suggesting that the decrease in the sedimentation rate observed in these experiments was the result of single-strand breaks in the DNA or alkaline-labile sites (19) rather than due to an enzymatic incision in the DNA during a repair event. DNA from cultures of 14C-labeled fibroblasts exposed to BP in the absence of hepatocytes showed no decrease in its sedimentation rate when compared to control (data not shown), suggesting that the fibroblasts themselves cannot activate BP to a DNA-damaging species.

The solubility of BP was investigated in the conditioned media collected as described in "Materials and Methods." There were clear differences in the solubility of BP in the media alone (Chart 4, triangles) or in the media conditioned by fibroblasts (Chart 4, solid circles), and in the media conditioned by hepatocyte fibroblast cocultures (Chart 4, open circles). The solubility of BP in media conditioned by hepatocytes alone was also determined, and the values were coincident with those of the coculture-conditioned media but, for clarity, were omitted from the chart. Conditioned media from the hepatocytes alone and the cocultures solubilized approximately 5-fold more BP than did the fibroblast-conditioned media at the lower concentrations but only slightly more at 100 μM BP. Approximately 50-fold more BP was solubilized by the coculture-conditioned media than was soluble in the media alone at 10 and 100 μM BP. We are uncertain if both the soluble and the insoluble BPs are available for metabolism by the hepatocytes, but if only the soluble form is metabolizable there was at least a 10-fold lower effective concentration of BP in the media than was expected from the amount of BP added to the cultures.
DISCUSSION

In our experiments, rat hepatocytes converted BP to metabolites which bound to and damaged hepatocyte and fibroblast DNA and induced a significant increase in the HPRT<sup>−</sup> mutation frequency in human fibroblasts cocultured with the hepatocytes. No DNA damage or mutations were observed when fibroblasts were exposed to BP in the absence of hepatocytes, indicating that both of these events were hepatocyte mediated. These results are in agreement with those in which BP was shown to be mutagenic to diploid human fibroblasts in a human carcinoma-derived cell line-mediated assay (3) and was mutagenic to Salmonella when activated by freshly isolated rat hepatocytes (17, 21). BP was also mutagenic to V79 cells in a BHK21 cell-mediated assay (35) but was inactive in a hepatocyte-mediated assay (29, 30). Reasons for the contrasting results between our present data and those reported previously (29, 30) are unclear. There are differences in the target cells for the mutagenesis assays (diploid human fibroblasts versus V79 cells). A possible explanation for the differences was that rat hepatocytes were releasing proximate carcinogens such as BP-7,8-dihydrodiol or 9-hydroxy-BP, which were further metabolized to the ultimate mutagens in the respective target cells. Human fibroblasts were shown to activate both BP-7,8-dihydrodiol and 9-hydroxy-BP to species which damaged DNA (36). V79 cells may lack the ability to metabolize the proximate carcinogens to their ultimate mutagenic species. Rat hepatocytes have been shown to release 9-hydroxy-BP and BP-7,8-dihydrodiol and other metabolites into the extracellular media in culture (12, 24, 37, 43). However, there was also an apparent release of "active" metabolites from hepatocytes in culture because BP was reported to bind to the extracellular DNA added to the media with the hepatocytes (37, 43). Comparisons of the extent of binding and the nature of the adducts would be useful for a resolution of the discrepancy between the V79 cell and the human fibroblast systems.

When hepatocyte fibroblast cocultures were exposed to 30 or 100 µM BP for 24 hr, there was a slight decrease in the sedimentation rate of both the hepatocyte and fibroblast DNA through 5 to 20% alkaline sucrose gradients when compared to control. These results are in agreement with other recent reports of small increases in strand breakage in cells exposed to activated BP (15, 46). Nordenskjold et al. (37) measured more strand breakage in the hepatocyte DNA than we report here, but our results are difficult to compare directly because of differences in experimental techniques and animals.

Even though 86 to 96% of the BP metabolites bound to the fibroblast DNA were due to the activation of BP by the hepatocytes (Chart 2), approximately 4 times more BP was bound to the fibroblast DNA (per µg of DNA) than was bound to the hepatocyte DNA when cocultures of the 2 cell types were exposed to a wide range of concentrations of [3H]BP. Those results are in agreement with our previous observations of the differences in the binding of hepatocyte and fibroblast DNA and were probably the basis for the greater amounts of unscheduled DNA synthesis measured in the fibroblasts than in the hepatocytes in cocultures after BP exposures (47). These data suggest that there was an apparent protection of the hepatocyte DNA from active metabolites of BP generated in its cytoplasm. Protection of the hepatocyte DNA could result from an efficient transport of BP metabolites out of the hepatocyte to limit the interaction with its own DNA and/or from an effective inactivation of reactive BP metabolites by pathways in the hepatocytes. Concerning the transfer of active metabolites of BP from the hepatocyte, the greater binding of BP to fibroblast DNA compared to hepatocyte DNA in the present experiments is consistent with the observations of Shen et al. (43) who observed that most of the DNA binding which occurred when DNA was added to the media surrounding hepatocytes in culture was on the extracellular DNA. The nature of the DNA adducts on the hepatocyte and fibroblast DNA are being investigated currently in our laboratory, but previous reports have indicated that extracellular DNA was modified by BP metabolites in a manner qualitatively identical to cellular DNA (37, 43).

Carriers may limit the interaction of BP metabolites with hepatocytic DNA. After i.p. injection of BP into rats, the kidney and lung concentrations of BP were found not to reflect the level of metabolizing enzymes but rather reflected the efficient transport from the liver of BP-protein complexes (42). Plasma lipoproteins have also been shown to transport BP and its metabolites in vivo (44), and rat hepatocytes excrete lipoproteins in culture (5). A secretion of lipoproteins by the hepatocytes may be responsible for the 5- to 50-fold-enhanced solubility of BP in the media conditioned by hepatocytes and hepatocyte fibroblast cocultures (Chart 4). BP metabolites are released from the liver in vivo. Recently, Chipman et al. (8) reported that hepatic venous blood contained unconjugated BP metabolites after an intraportal infusion of [3H]BP and speculated that reactive metabolites produced in the liver could reach extrahepatic sites. Our in vitro data and those by others (8, 22, 24, 37, 42, 43) suggest that the liver may be a source of proximate and ultimate carcinogenic metabolites of BP with the potential to induce extrahepatic carcinogenesis. The extrahepatic efflux of active metabolites is not limited to BP. Tumors of the nonparenchymal cells of the liver are thought to be induced by the passage of active metabolites generated in parenchymal cells after the administration of dimethylnitrosamine (1, 49) and dimethylhydrazine (31).

In addition to the release of BP metabolites from the hepatocytes, an efficient inactivation of BP within the hepatocytes would also limit the interaction of BP with the hepatocytic DNA. Active metabolites of BP are inactivated by nonspecific reactions with nucleophiles and by specific enzymatic mechanisms.

Chart 4. Solubility of BP in conditioned media. The indicated molar concentrations of BP were made up in media conditioned by a 45-hr exposure to hepatocyte-fibroblast cocultures (C), fibroblasts alone (W), or culture dishes with no cells (A). Insoluble BP was separated from solubilized BP by centrifugation at 100,000 × g for 1 hr. The amount of BP remaining in solution was compared to reference total values taken immediately before the centrifugation of the media. Bars, S.E.
BP binding to DNA was shown to be affected markedly by the concentration of cofactors for conjugation such as UDP-glucuronic acid and glutathione (9, 18, 34, 37, 43). Different metabolites of BP may require different modes of inactivation. The binding of 9-hydroxy-BP to DNA was inhibited by the addition of weak nucleophiles such as albumin, whereas the inactivation of BP-7,8-dihydrodiol was inhibited only by a glutathione-dependent reaction with cystolic components (37).

Carcinogen activation and detoxification may also occur at the level of the nuclear membrane (51). The nuclear envelope is a specialized extension of the endoplasmic reticulum and contains many of the same xenobiotic metabolizing systems as the endoplasmic reticulum, notably cytochrome P-450, NADPH-cytochrome c reductase, epoxide hydratase, and UDP-glucuronosyltransferase (16, 25, 38, 51, 53). Key work by Viviani and Lutz (51) showed that binding of BP to rat liver DNA in vivo was regulated by the balance of microsomal and nuclear aryl hydrocarbon hydroxylase activity. These workers were able to show that the induction of nuclear aryl hydrocarbon [benzo(a)pyrene] hydroxylase had a negative effect on the total binding of BP to DNA, rather than a positive effect as had been expected. The basis for the protective effect offered by the nuclear membrane from BP activated in the cytoplasm was suggested to be due to the detoxification of BP metabolites by epoxide hydratase and conjugation pathways known to be present in the nuclear envelope (25, 38, 51, 53). Carcinogen inactivation at the level of the hepatocyte nuclear membrane could contribute to the difference that we observed between the relative binding of BP to hepatocyte and fibroblast DNA and may also help explain previous reports of differences in the binding of BP metabolites to nuclear DNA and cytoplasmic mitochondrial DNA (4).

The facility with which active metabolites of BP leave the hepatocytes (37, 43) and the relative protection of the hepatocyte DNA from alkylation may in part be responsible for the lack of carcinogenicity of BP for the adult rat liver. The major DNA-bound form of BP in the liver (2, 52) is the same adduct thought to be responsible for the tumorigenicity of BP in several tissues sensitive to the carcinogenic effect of BP (3, 46, 50, 55). No unique metabolite or DNA-bound form of BP has ever been found in tissue sensitive to BP which has not also been found in the liver. Since the adduct profile and the metabolites of BP generated by the liver are qualitatively the same as in sensitive tissues, quantitative differences in binding and other factors including DNA repair and the rate of cell division are believed to be of crucial importance to tumor formation (2, 10, 14, 32). Covalently bound products of BP were investigated in Swiss mice, a strain sensitive to BP-induced carcinogenesis, and in Wistar rats, which are resistant to BP (14). Mice and rats had qualitatively the same adducts; however, total binding to the rat skin was 3-fold lower than to mouse skin. We have found differences of the same magnitude in the relative binding of BP to hepatocyte and fibroblast DNA.

Another observation relevant to the lack of carcinogenicity of BP for the liver was that BP exposure resulted in the production of few single-strand breaks in the DNA. The presence of single-strand breaks in the livers of 2-acetylaminofluorene (N-2-fluorenylacacetamide)-treated rats was correlated with an increase in the tumor incidence. A much lower tumor incidence was associated with a selenium-supplemented diet, which also prevented the induction of single-strand breaks in the liver (54). The DNA in the livers of both groups of rats was covalently modified by 2-acetylaminofluorene (N-2-fluorenylacacetamide) to the same extent, suggesting that the induction of single-strand breaks in the liver may be an important component to eventual tumor development.

In the present report, primary cultures of rat hepatocytes cocultured with human fibroblasts were used to investigate the results of the intercellular transfer of BP metabolites. Metabolites of BP activated by hepatocytes were effectively passed to the fibroblasts and were partially prevented from interacting with the hepatocytic DNA. The relative protection of the hepatocytic DNA from BP activated in the cytoplasm of the hepatocyte may be important to the known lack of carcinogenicity of BP for the liver in vivo.

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