Production of oxidative DNA damage during the metabolic activation of benzo[a]pyrene in human mammary epithelial cells correlates with cell killing

(oxygen radicals/radical scavengers/immunoassay/DNA damage/DNA repair)

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Communicated by Melvin Calvin, March 18, 1988 (received for review October 6, 1987)

ABSTRACT We have studied the generation of reactive oxygen species during the metabolism of a carcinogen, benzo[a]pyrene, by human mammary epithelial cells. We have quantitated the production of one type of oxidative DNA damage, thymine glycols, by using a monoclonal antibody specific to this base modification. Thymine glycols were produced in DNA in a dose-dependent manner after exposure of human mammary epithelial cells to benzo[a]pyrene. The number of thymine glycols formed in the DNA was similar to that of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene covalently bound to the DNA. Exposure of cells to the carcinogen in the presence of superoxide dismutase, which reduces superoxide anions, inhibited the production of thymine glycols and increased cell survival but had little effect on adduct formation. At equitoxic doses, ≈ 10 -fold more thymine glycols were formed after exposure to benzo[a]pyrene than to γ -irradiation. Thymine glycols, produced by either agent, were efficiently removed from the DNA of the cells. Since thymine glycols represent only a portion of the oxidative damage possibly produced, our results indicate that the total amount of oxidative damage induced during the exposure of human mammary epithelial cells to benzo[a]pyrene greatly exceeds the amount produced by direct adduct formation and that this indirect damage plays an important role in the cytotoxicity of benzo[a]pyrene.

There is increasing evidence implicating the involvement of free radicals, particularly those derived from molecular oxygen, in many stages of chemical carcinogenesis (1, 2). Active oxygens and the ensuing lipid peroxidations could affect carcinogenic processes in at least two ways: (i) by causing chromosomal damage and rearrangements and (ii) by modulating cell growth and differentiation through epigenetic mechanisms. For example, it is known that hydroxyl radicals are responsible for 60–70% of DNA strand breaks, chromosomal aberrations, mutations, and cell killing produced by ionizing radiation (3).

Benzo[a]pyrene (B[a]P) is a potent mutagen (4) and carcinogen (5). Its metabolism and subsequent binding to DNA has been extensively studied in cell culture (6, 7). We have previously shown (8, 9) that human mammary epithelial cells (HMEC) in culture are capable of metabolizing B[a]P. The metabolic pattern of the epithelial cells and the production of DNA adducts were characteristic of the preferential formation of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P diol epoxide) as the ultimate carcinogen. A major pathway of B[a]P metabolism also leads to the formation of free radicals that are reactive with DNA, indirectly producing damage (10–12). To understand the role in carcinogenesis of damage induced by chemical carci-

gens, it is important to identify the primary DNA lesions produced directly by adduct formation and indirectly by free radicals.

One product that is formed in DNA as a consequence of chemical oxidation or ionizing radiation is thymine glycol (13, 14). A sensitive immunoassay for thymine glycols has been developed with a monoclonal antibody to this product in DNA (15). By using this methodology, thymine glycols have been detected in DNA after treatment of cultured human fibroblasts with ionizing radiation, 254-nm ultraviolet light, and an active metabolite of the carcinogen 2-naphthylamine (16, 17). We have used this method to determine whether reactive oxygen species are being produced during the metabolism of B[a]P by HMEC and what role these reactive oxygen species play in cellular toxicity.

Our results show that: (i) thymine glycols are produced in DNA in a dose-dependent manner at levels comparable to the amount of B[a]P diol epoxide covalently bound to DNA adducts after exposure of HMEC to B[a]P; (ii) the production of thymine glycols, but not that of B[a]P adducts, is reduced if superoxide dismutase (SOD) is present at the time of treatment; (iii) survival is increased if SOD is present during treatment with B[a]P; (iv) at equitoxic doses, 10-fold more thymine glycols are produced after B[a]P treatment than after ionizing radiation.

MATERIALS AND METHODS

Chemicals. [^3H]B[a]P (17.4 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham and unlabeled B[a]P was from Aldrich. Both substrates were further purified from silica gel plates by thin-layer chromatography and their purity was checked by high-pressure liquid chromatography (18). The purity of the B[a]P used in these experiments was $>98\%$ ($<0.8\%$ as the quinone). Catalase and SOD were obtained from Sigma.

Cell Culture. The mammary epithelial cells were isolated and grown as described (19, 20). All experiments were performed with normal HMEC from one specimen, no. 184, derived from reduction mamoplasty tissue. Cells were grown in the serum-free medium MCDB170, which supports long-term active growth of normal HMEC (21).

Exposure to B[a]P. Actively proliferating cells, between passages 7 and 10, were exposed to B[a]P as described by Bartley and Stampfer (18), in medium containing 0.1% dimethyl sulfoxide for 4 hr at 37°C. For repair experiments, the cultures were washed with phosphate-buffered saline (PBS) and incubated in medium for various time intervals at 37°C before lysis. For experiments without a posttreatment

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Abbreviations: B[a]P, benzo[a]pyrene; B[a]P diol epoxide, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; HMEC, human mammary epithelial cells; SOD, superoxide dismutase.

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incubation, the cultures were washed twice with PBS and harvested by trypsinization. In some experiments, aliquots of the cells were used to assay colony-forming ability with the remainder of the cells stored as a frozen pellet prior to extraction of the DNA.

Exposure to Ionizing Radiation. Cell cultures were irradiated at room temperature with a ^{60}Co source at a dose rate of 95 rad/min (1 rad = 0.01 Gy).

Colony-Forming Ability. For survival studies, treated cell cultures were trypsinized and reseeded into at least six 60-mm culture dishes per experimental condition. At the end of 6–9 days, cells were stained with methylene blue and colonies that contained >50 cells were counted as survivors. Colony-forming efficiency for control cultures ranged from 14% to 52%.

DNA Isolation. Cell pellets were taken up and lysed in 10 mM Tris-HCl/1 mM EDTA, pH 8/0.1% sodium dodecyl sulfate and incubated first with RNase A (0.1 mg/ml) for 1 hr and then with proteinase K (0.1 mg/ml) for 1 hr at 37°C. The DNA was purified from the cell lysate by sequential extractions with phenol, phenol/chloroform/isoamyl alcohol (24:24:1), and chloroform/isoamyl alcohol (24:1), followed by precipitation in 70% ethanol. The DNA was redissolved in PBS and the concentration and purity were determined spectrophotometrically by absorptions at 260 and 280 nm. The extent of adduct formation with DNA was determined by counting an aliquot of the DNA sample in PCS (Amersham) with a Packard Tri-Carb scintillation counter. The concentration of total B[a]P adducts per 10^6 base pairs of DNA was then computed from the specific activity of the ^3H B[a]P preparation. To verify that the radioactive label bound to the DNA accurately reflected the level of B[a]P adducts, the DNA was digested to its constituent nucleosides, the nucleosides covalently bound to B[a]P metabolites were separated from free nucleosides by chromatography on Sephadex LH 20, and the adducted nucleosides were identified by high-pressure liquid chromatography (18).

ELISA. The ELISA for quantitation of thymine glycols in DNA was carried out as described by Leadon and Hanawalt (15) with the monoclonal antibody designated GB₁₀/C₅. This antibody did not cross-react with DNA adducts produced by directly modifying DNA *in vitro* with B[a]P diol epoxide.

RESULTS

In these studies, we used a sensitive immunoassay to determine the frequency of thymine glycols in DNA. A constant amount of immobilized OsO_4 -modified DNA and various amounts of a competitor in solution were reacted with a fixed amount of the antibody. The amount of the antibody bound to the immobilized DNA was then determined and compared to the amount bound in the absence of the competitor. We used OsO_4 -modified DNA in the assay because OsO_4 selectively oxidizes the 5,6 double bond of thymine to produce the glycol but does not react at all with other bases (22). The ELISA was calibrated by using serial dilutions of radioactively labeled DNA modified by OsO_4 as the standard. The thymine glycol content of the standard DNA was determined chromatographically (15).

Production of Thymine Glycols in HMEC. HMEC were exposed to various doses of ^3H B[a]P for 4 hr at 37°C. DNA was extracted from these cultures, during which time unbound carcinogen was also removed, and the DNA was used as a competitor in the ELISA and for determination of total DNA adducts. The extent of inhibition of antibody binding to the immobilized DNA was proportional to the concentration of B[a]P to which the cells were exposed. The formation of thymine glycols in HMEC was proportional to the concentration of B[a]P up to 5 $\mu\text{g}/\text{ml}$ (Fig. 1). The production of DNA adducts, as determined by the radioactivity bound to

FIG. 1. Production of B[a]P diol epoxide-DNA adducts and thymine glycols in DNA from B[a]P-treated HMEC cultures. Cells were exposed to various concentrations of ^3H B[a]P for 4 hr. Total B[a]P adduct frequencies were derived from the ^3H radioactivity in the purified DNA and from the specific activity of the ^3H B[a]P preparation. Thymine glycol frequencies were determined in a competitive ELISA by reacting antibody (1:1000 dilution) with 50 ng of OsO_4 -oxidized DNA (0.04% thymine glycols). b.p., Base pairs.

the DNA, was also proportional to the concentration of B[a]P to which the cells were exposed (Fig. 1). The number of thymine glycols formed was of the same order of magnitude as B[a]P diol epoxide covalently bound to the DNA. Metabolic activation of B[a]P was required for the formation of thymine glycols since directly modifying DNA by exposure to B[a]P diol epoxide *in vitro* did not produce oxidative DNA damage (data not shown).

It was previously shown that the presence of catalase and SOD reduced the formation of both thymine glycols and strand breaks in DNA treated *in vivo* with an active metabolite of the carcinogen 2-naphthylamine (16, 23). To investigate the indirect effects of B[a]P metabolism on the formation of DNA damage and on cellular toxicity, HMEC cultures were exposed to B[a]P in the absence and presence of either catalase or SOD or both catalase and SOD. When cultures were exposed to B[a]P (2.5 $\mu\text{g}/\text{ml}$), ≈ 9 thymine glycols per 10^6 base pairs were formed (Table 1). Incubation of treated cultures in the presence of various concentrations of SOD reduced the amount of thymine glycols in a dose-dependent manner. Exposure of HMEC to B[a]P in the presence of 250 μg of SOD decreased the amount of thymine glycols formed by >90%. In contrast, DNA adduct formation was reduced by only 11%. Catalase had little effect on the production of thymine glycols. Therefore, while SOD did not significantly inhibit the metabolism of B[a]P as indicated by the amount of B[a]P diol epoxide covalently bound to the DNA, it did reduce the amount of damage indirectly produced by the generation of active oxygen species.

When cultures were exposed to B[a]P (2.5 $\mu\text{g}/\text{ml}$), cell survival was reduced to 29% of the mock-treated controls (Table 1). Incubation of cultures with B[a]P in the presence of various amounts of SOD increased survival significantly. In the presence of SOD (250 $\mu\text{g}/\text{ml}$), survival was similar to the untreated controls. Catalase, however, did not increase survival when incubated with B[a]P, and when used in combination with SOD, it actually decreased the apparent protective effect of SOD. Catalase by itself was not toxic to the cells. Thus, the toxicity to HMEC of a short-term exposure to B[a]P appears to be correlated to the production of oxidative damage rather than to direct carcinogen binding to the DNA.

Table 1. Effect of SOD and catalase on production of thymine glycols, production of B[a]P diol epoxide-DNA adducts, and survival in human mammary epithelial cells exposed to B[a]P

Treatment	Thymine glycols per 10 ⁶ base pairs	B[a]P diol epoxide adducts per 10 ⁶ base pairs	% survival
None	—	—	100 ± 4
B[a]P (2.5 µg)	9.2 ± 0.6	8.1 ± 0.4	29 ± 8
B[a]P (2.5 µg) + SOD (10 µg)	8.9 ± 0.4	8.7 ± 0.6	17 ± 1
B[a]P (2.5 µg) + SOD (50 µg)	6.8 ± 0.4	7.3 ± 0.5	47 ± 8
B[a]P (2.5 µg) + SOD (100 µg)	1.8 ± 0.4	6.4 ± 0.5	75 ± 2
B[a]P (2.5 µg) + SOD (250 µg)	0.6 ± 0.2	7.2 ± 0.3	96 ± 4
B[a]P (2.5 µg) + catalase (100 µg)	8.4 ± 0.6	ND	17 ± 2
B[a]P (2.5 µg) + catalase (100 µg) + SOD (100 µg)	2.1 ± 0.3	ND	58 ± 4

Results are expressed as the average ± SEM of 3–12 experiments. ND, not determined.

Comparison of the Production and Repair of Thymine Glycols After Exposure to B[a]P or γ -Rays. Most of the biological effects of ionizing radiation are mediated by reactive oxygen species. Since oxidative DNA damage was also being produced during the metabolism of B[a]P, we compared this to the production of thymine glycols in HMEC after an equitoxic dose of γ -rays (Table 2). Exposure of cultures to 500 rads reduced the survival to 26% of controls compared to 29% for B[a]P (2.5 µg/ml). However, there was at least a 10-fold greater yield of thymine glycols after exposure to B[a]P. The kinetics of repair of thymine glycols was compared after treatment with either 400 rads of ionizing radiation or B[a]P (1 µg/ml). After either treatment, the cells exhibited an initial rapid removal of thymine glycols, with $\approx$ 50% removal by 45 min.

DISCUSSION

We have found that during the metabolic activation of B[a]P by HMEC, reactive oxygen species are formed that result in the production of indirect damage in the DNA. One type of indirect damage was measured, thymine glycol. This damage was produced in quantities equal to or greater than the level of direct DNA adducts after an acute exposure to the carcinogen. Similar results were reported by Ide *et al.* (12) for production of damage to the methyl group of thymine in human lung cells after longer (40 hr) exposures to B[a]P. Since thymine glycols and thymine methyl damage represent only a fraction of the indirect damage possibly produced (24), the amount of total indirect damage induced during exposure to B[a]P greatly exceeds that of direct adduct formation.

The indirect action of B[a]P may be mediated by active oxygen species and other free radicals that are produced during the autoxidation of a primary metabolite of B[a]P, 6-hydroxy-B[a]P (25). In a system in which two types of active oxygens, hydrogen peroxide and superoxide anion, are formed, a further reaction leading to the generation of a more reactive species of free radical, the hydroxyl radical, is expected via a Haber-Weiss type of reaction (26). The addition of these hydroxyl radicals across the 5,6 double bond of thymine in the presence of oxygen can result in the formation of the glycol, 5,6-dihydroxydihydrothymine (27). Incubation of HMEC cultures with SOD, which reduces

superoxide anions, in the presence of B[a]P decreased the amount of thymine glycols formed and increased survival to near untreated levels. However, the amount of total B[a]P adducts formed was unchanged. Therefore, the cytotoxic effects of B[a]P appear to be related to the generation of reactive oxygen species and indirect damage.

Incubation of the cells with catalase, which breaks down hydrogen peroxide, in the presence of B[a]P did not increase survival or reduce the amount of thymine glycols. This result is in contrast to previous studies in which both SOD and catalase decreased the amount of indirect DNA damage produced by the autoxidation of the carcinogen *N*-hydroxy-2-naphthylamine (16, 23). These differences may be reconciled in part by the fact that B[a]P may be a "membrane-active agent" (28). B[a]P has been shown to stimulate prostaglandin production in cultured dog kidney cells (29). The synthesis of prostaglandins occurs via the arachidonic acid cascade, and it has been proposed that this pathway produces active oxygen at the level of the cellular membrane (28). Since exogenous SOD and catalase are not taken up by the cell, SOD may exert its protective effect by breaking down superoxide anions extracellularly and in the proximity of the membrane. Catalase, however, may not be accessible to the intracellular location where hydrogen peroxide is being formed during the metabolism of B[a]P. Autoxidation of *N*-hydroxy-2-naphthylamine, on the other hand, can occur both extra- and intracellularly, and therefore both SOD and catalase have access to the active oxygen species formed during this process.

Since most of the biological effects of ionizing radiation are mediated through the formation of reactive oxygen species, the number of thymine glycols formed after exposure of HMEC to B[a]P was compared to the number formed after an equitoxic dose of γ -rays. There were at least 10-fold more thymine glycols formed after exposure to B[a]P than to γ -rays. Therefore, even though reactive oxygen species can mediate the formation of indirect DNA damage by both agents, the spectrum of damage induced by the two agents is probably different—e.g., the formation of strand breaks is probably proportionally greater following ionizing radiation than following B[a]P metabolism. In addition, the repair pathway for the removal of thymine glycols is apparently not saturated even with the higher initial levels found after treatment with B[a]P since the percentage removed was similar with both agents.

The significance of the production of thymine glycol and other types of indirect damage after treatment with a chemical carcinogen is as yet unclear. While covalent binding of active metabolites of a carcinogen with DNA appears to be an obligatory step in the production of a tumor, it also appears to be insufficient for malignant cell transformation. For example, the rate of skin tumor production by B[a]P diol epoxide without promotion by 12-*O*-tetradecanoylphorbol 13-acetate is extremely low compared with that of the parent

Table 2. Comparison of thymine glycol production after exposure of human mammary epithelial cells to ionizing radiation and B[a]P

Treatment	% survival	Thymine glycols per 10 ⁶ base pairs
None	100 ± 4	—
Irradiation (500 rads)	26 ± 5	0.9 ± 0.2
B[a]P (2.5 µg)	29 ± 8	9.2 ± 0.6

Results are expressed as the average ± SEM.

B[a]P, in spite of significant ability of the former to bind to DNA (30). Our results are consistent with the concept that complete carcinogens exert their biological effects by both the direct DNA binding that is equated with the initiation step and the production of indirect damage—e.g., via active oxygens—that is associated with the promotion phase (28).

We thank Dr. Tracy Yang for assistance in irradiating the cells, Linda Hayashi and Kari Koszdzin for expert technical assistance, and Dr. Priscilla Cooper for helpful discussions. This work was supported by Public Health Service Grants CA24844, CA38889, and CA40453 awarded by the National Cancer Institute, and by the Office of Health and Environmental Research, Office of Energy Research, U.S. Department of Energy Contract DE-AC03-76SF00098.

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