DNA Damage by Benzo(a)pyrene in Human Cells Is Increased by Cigarette Smoke and Decreased by a Filter Containing Rosemary Extract, Which Lowers Free Radicals

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Abstract

We found previously that the human lung benzo(a)pyrene (BP)–7,8-diol–9,10-epoxide–N²-deoxyguanosine (BPDE-dG) adduct concentrate in the target bronchial cells. This adduct is now considered to be critical event in tumorigenesis by BP. In this study, we investigate the contribution of cigarette smoke on the BPDE-dG formation. In a cell-free system, the amount of (−)-anti-BPDE-dG adduct increased linearly with concentration of cigarette smoke in the presence of (+)-BP-7,8-diol. Catalase and superoxide dismutase inhibited its formation by >80%. When MCF-7 cells were treated for 2 hours with the (+)-BP-7,8-diol, cigarette smoke increased dose-dependently the formation of (−)-anti-BPDE-dG and decreased the cytochrome P450 (CYP)–dependent formation of (−)-r-7,8-dihydroxy-c-9,10-oxo-7,8,9,10-tetrahydro-BP the adduct. Then, cells were treated for up to 1 day with BP and then exposed for 2 hours with cigarette smoke. During these 2 hours, there are twice the increase in the adduct formation in cells treated with cigarette smoke compared with levels in nontreated cells due to CYP activity. Thus, cigarette smoke containing reactive oxygen species may activate the second step of BP metabolic way, leading to the formation of BPDE-dG adduct. Cigarette smoke thus seems may be in part responsible for the formation of the critical lung tumorigenic adduct. Finally, modified cigarette filter containing rosemary extract decreases by >70% of the BPDE-dG adducts level due to the cigarette smoke in MCF-7 cells. This approach may lead to decreasing lung cancer risk in addicted smokers. (Cancer Res 2006; 66(24): 11938-45)

Introduction

Cigarette smoking is causally associated with a large number of human cancers (1). Tobacco use is by far the most widespread link between exposure to known carcinogens and death from cancer and is therefore a model for understanding mechanisms of cancer induction. Benzo(a)pyrene (BP) is a highly carcinogenic polycyclic aromatic hydrocarbon (PAH) present in emission exhausts, in charbroiled food, and in small quantity of cigarette smoke (2–5), typically <10 ng/cigarette (6). BP is 1 of >60 carcinogens in cigarette smoke that is involved in the etiology of lung cancer (7). It is metabolically activated into BP-7,8-diol–9,10-epoxide (BPDE), which reacts with DNA predominantly at the N² position of guanine to produce primarily N²-guanine lesions [e.g., BPDE-N²-deoxyguanosine (BPDE-dG) adduct; ref. 8]. The presence of BPDE-DNA adducts in human tissues has been conclusively established (9) and BPDE-dG adduct concentrated exclusively in bronchial cells and thus implicated in the initiation of human lung cancer (10). Although considerable evidence implicates BP as important causative agent of smoking-related cancers, its role is clearly not exclusive.

This carcinogen is metabolized by phase I enzymes to a large number of metabolites, including phenols, arene oxides, quinones, dihydrodiols, and diol epoxides (11). An overview of BP metabolic way leading to the formation of (−)-anti-BPDE-dG adduct is presented in Fig. 1.

The ultimate carcinogen (+)-r-7,8-dihydroxy-t-9,10-oxo-7,8,9,10-tetrahydro-BP [(+)-anti-BPDE] is formed from BP by both two rounds of cytochrome P450 (CYP)–mediated oxidation. The first step of this oxidation leads preferentially to (−)-trans-7,8-dihydroxy-7,8-dihydro-BP [(−)-BP-7,8-diol]. The diol is further oxidized primarily to the highly mutagenic (+)-anti-BPDE (12). Numerous studies have clearly identified the [(+)-anti-BPDE] as the primary carcinogenic metabolite of BP exhibiting enhanced mutagenic activity in vitro and in vivo (13–17). Most previous studies of genetic variation in metabolism of lung carcinogens have focused on metabolic activation by various CYPs, although expression of these enzymes in lung is generally low (18). The activation of BP-7,8-diol by lung epithelial cells is not caused solely by classic CYP/glutathione S-transferase (GST)–dependent biotransformation processes but also involves several metabolic routes other than CYPs. These include lipoxygenase, lipid peroxidation products, and peroxidase-dependent pathways (19–23), cyclooxygenase (COX)-1 and COX-2 (24).

Increasing evidence suggests the causal significance of tobacco-free radicals in lung cancer induction in smokers (6, 7, 25–27). Each puff of smoke contains >10 trillion free radicals, which may contribute to both tumor initiation as well as promotion (28, 29) of various forms of human cancer caused by repeated attacks from reactive oxygen species (ROS) on cellular macromolecules. The major free radical species was postulated to be an equilibrium mixture of semiquinones, hydroquinones, and quinones (25). It is suggested that this free radical complex causes redox cycling that generates superoxide anion from molecular oxygen and leads to the formation of hydrogen peroxide (H₂O₂) and hydroxyl radical (27). These reactive species cause DNA nicking (27) and single-strand breaks in DNA of cultured rodent and human cells (30, 31). Quinone-associated redox cycling may also be involved in these effects; hydroquinone and catechol are believed to play a major role.

Our study was designed (a) to determine the relative contribution of ROS in the cigarette smoke on the activation of BP-7,8-diol in comparison with CYP and (b) to apply the method used in this study...

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to answer the following questions: (i) does the ROS of cigarette smoke promote the carcinogenic process by contributing to the metabolism of BP-7,8-diol resulting in an increase in the formation of the critical lung BPDE-dG? and (ii) does the ROS of cigarette smoke promote the carcinogenic process by contributing to the metabolism of BP-7,8-diol resulting in an increase in the formation of the critical lung BPDE-dG? and (ii) can a filter containing a scavenger of cigarette-free radicals affect the formation of BPDE-dG?

Materials and Methods

**Chemicals.** Proteinase K (EC 3.4.21.64, from *Trichoderma album*) was purchased from Sigma (St. Louis, MO) and RNase T1 (EC 3.1.21.3, from *Aspergillus oryzae*) and RNase (DNase-free, heterogeneous mixture of RNases from bovine pancreas) was obtained from Roche Diagnostics (Meylan, France). PBS adjusted at pH 7.4 contained 3.0 mmol/L KCl, 1.5 mmol/L KH₂PO₄, 140 mmol/L NaCl, and 8.0 mmol/L Na₂HPO₄. High-performance liquid chromatography (HPLC)-grade water, methanol, diethyl ether, and ethanol (Merck, for spectroscopy grade) were obtained from VWR International (Fontenay-sous-Bois, France). If not stated otherwise, the other chemicals were purchased from Sigma (Usine d’Abeau Chesnes, France). All BP metabolite standards were obtained from National Cancer Institute, Chemical Carcinogen Reference Standard Repository Midwest Research Institute (Kansas City, MO).

**Apparatus.** HPLC was carried out with Agilent high-pressure isocratic and gradient systems (Massy, France) equipped with a Shimadzu RF-10AXL integrator. (Champs-sur-Marne, France) fluorescence detector linked to Agilent integrator.

**Preparation of cigarette smoke/PBS solution.** Smoking was done according to Pryor et al. (25) without the Cambridge filter. Essentially, the same smoke-collecting method has been used earlier by Nakayama et al. (30, 31). The smoke from burning 8 cm of one cigarette (Marlboro) during 3.8 minutes with the help of constant vacuum generated from a water pump was bubbled through 10 mL PBS solution, which traps both gas-phase and tar cigarette smoke chemicals. As there were no water-insoluble tar compounds present on the walls of the wash bottles, a major part of the water-soluble compounds from the smoke of a single cigarette was contained in the 10 mL PBS solution. This aqueous solution named cigarette smoke solution (CSS) was reacted immediately with exogenous DNA or added to MCF-7 cells in culture in the presence of BP or its proximate metabolite (+)-BP-7,8-diol. Different dilutions of CSS were used (see below).

**Incorporation of a powder extract into the cigarette filter.** The filter of the conventional cigarette was removed and 40 mg of a rosemary powder extract kindly provided by Biosynthetic Co. (Paris, France) and prepared according to Dr. Imen Emami (Biosynthetic) patent process (32) was introduced in the place liberated by the removal of the filter near the cigarette itself. After this operation, the filter was reinstalled. The effect of this filter was evaluated by mass spectrometry (MS; ref. 33). Briefly, the cigarette smoke was bubbled in an organic solution containing 3,3,5,5-tetramethyl-1-pyrroline-N-oxide (TMPO), a spin trap adduct. The amount of hydroxyl radical adduct was then quantified using liquid chromatography (LC), MS.

**Reaction of exogenous DNA with (+)-BP-7,8-diol in the presence of diluted CSS.** Two milliliter calf thymus DNA (3 mg/mL) was added to 5 mL CSS diluted 20 times and reacted for 2 hours at room temperature with (+)-BP-7,8-diol (final concentration, 3.6 μmol/L) according to the following reaction:

\[
\text{DNA} + [(+)-\text{BP-7,8-diol}] + \text{CSS} \rightarrow (\text{anti})-\text{anti-BPDE-1}^{2}-\text{dG}
\]

The level of the resulting (anti)-BPDE-dG adduct was measured (see below). As control, an experiment without CSS was done.

**Cell culture conditions and treatment.** The human mammary carcinoma cell line MCF-7 was grown in 150-cm² cell culture flasks in a total volume of 20 mL EMEM supplemented with 10% FCS, 15 mmol/L HEPES buffer, and antibiotics (200 units/mL penicillin, 200 μg/mL streptomycin, and 25 μg/mL ampicillin). Cells were maintained and treated at 37°C in 5% CO₂/95% air atmosphere.

After MCF-7 cells had covered 90% of the surface area of the flasks, (2–3 days after splitting of a confluent culture), the medium was replaced with 20 mL of fresh medium containing 10% serum. Twenty four hours later, near-confluent cells (e.g., >90% of cells in G₀-G₁ phase) were treated with DMSO alone or with carcinogen (see below) dissolved in DMSO and CSS/PBS (CSS, see above). The final concentration of DMSO did not exceed 0.1% of the total incubation volume. Control samples included in each incubation set were treated with DMSO alone.

(a) Treatment of MCF-7 cells with (+)-BP-7,8-diol and cigarette smoke. Cells were treated for 2 hours with (+)-BP-7,8-diol (0.2 μmol/L) alone or in the presence of different dilutions of CSS. The (+)-BP-7,8-diol was activated by ROO⁺ generated from the cigarette smoke and cell CYPs to form (anti)-anti-BPDE-dG and (anti)-syn-BPDE-dG, respectively.

(b) Time/dose exposure experiments with BP. To characterize time/dose exposure to BP and BPDE-dG level, cells (10 × 10⁶/150-cm² flask, total volume of 20 mL) were treated with medium containing final concentration of 1.25, 2.5, and 5.0 μmol/L each for 6, 12, 18, and 24 hours (two flasks/dose/time point). BPDE-dG adduct formed in the cells increase linearly in a dose- and time-dependent manner as also shown by others (34). Based on the results obtained, we chose 2.5 mmol/L as working concentration for BP.
(c) Treatment of MCF-7 cells with BP and cigarette smoke. To see the effect of cigarette smoke concentration, the cells were treated with different CSS dilutions (1:79, 1:39, 1:19, and 1:9 v/v). Based on the results obtained from this experience, we chose 1:19 (v/v) dilution as working cigarette smoke concentration. Thus, the cells (see above, “cell culture and treatment”) were treated with BP (2.5 μmol/L) for 12 hours (group A) or 18 hours (group B) and then exposed during the last 2 hours to standard CSS or rosemary-filtered CSS (dilution, 1:19 v/v); cells treated only with BP during 14 hours (respectively 20 hours) were used as control.

All incubation sets were repeated two to three times with duplicate samples. At the end of treatment, cells were examined microscopically for morphologic changes and then harvested by trypsinization with 0.05% trypsin-EDTA (0.05% trypsin, 0.14 mol/L NaCl, 3 mol/L KCl, 0.1 mol/L Na2HPO4, 1.5 mol/L K2HPO4, and 0.5 mol/L EDTA). After addition of an equal volume of medium containing 10% FCS, the cells were centrifuged at 1,000 × g and washed thrice with PBS, and the cell pellet was then stored frozen at −20°C. The viability of the cells treated with BP and cigarette smoke or (+)-BP-7,8-diol and cigarette smoke was roughly 90% at the time of harvesting as determined by a trypan blue exclusion assay. The doses used did not show any cytotoxicity as measured by lactate dehydrogenase

DNA preparation and hydrolysis. DNA isolation from MCF-7 cell pellets was carried out by treatment with RNase, proteinase K, salting procedure (35), and chloroform. Briefly, the cell pellets were resuspended in EDTA-SDS buffer [10 mmol/L Tris buffer, 1 mmol/L Na2EDTA, 1% SDS (w/v; pH 8.0)] incubated for 1 hour at 37°C with RNase T1 (2,000 units/mL) and RNase A (100 μg/mL; DNase-free) on a shaker (100 rpm). Then, proteinase K (300 μg/mL) was added and the incubation was continued overnight at 37°C. After digestion, 6 mol/L NaCl was added to have final concentration of 1 mol/L followed by a centrifugation at 10,000 × g. DNA in the supernatant was precipitated with two volumes of ethanol, washed with 70% ethanol, ether twice, then dried, and dissolved in 10 mol/mL Tris buffer. Again, RNase A (100 μg/mL) and RNase T1 (2,000 units/mL) were added and the solution was incubated at 37°C for 1 hour followed by proteinase K (100 μg/mL) for another 2 hours at 37°C. The solution was extracted once with chloroform and centrifuged and the solution was made of 1 mol/L NaCl. DNA was precipitated with two volumes of cold ethanol. The portion of DNA to be hydrolyzed was rinsed with 100% ethanol to remove unbound BP-tetrols. The DNA, free of unbound BP-tetrols, was dissolved in water and the DNA concentration was determined by A260 nm. The purity was ascertained by the ratios at A260/A280 and A260/A230. The amount of DNA for analysis was hydrolyzed as described previously (36, 37) by incubation at 90°C for 4 hours in a final concentration of 0.1 N HCl. This releases tetrads from BPDE-DNA adducts with >90% recovery (36). The volume of the hydrolysate for injection was made 700 μL containing 5 to 10 μg DNA.

Determinations of BPDE-N2-DG adduct level. The adduct levels were determined by HPLC-fluorescence detection as described previously (36, 37) using r-7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro-BP (BP-tetrol II-1) as an internal standard (38). The hydrolysate was loaded onto a precolumn containing 5 μm C18 reverse-phase material (Nucleosil 100) equilibrated with 10% methanol and washed for 20 minutes with 12 mL methanol (10%). Subsequently, the precolumn was switched by a Valco Instruments switching valve to flow over a 4.6 mm × 25 cm 5 μmol/L C18 reverse-phase (Nucleosil 100) analytic column (Alltech, Templemars, France). The products obtained by hydrolysis were eluted with the following methanol/ H2O gradient: 50%, 0 to 17 minutes; 50% to 60%, 17 to 32 minutes; 60% to 42 minutes; and 60% to 100%, 42 to 57 minutes. Retention times of the BP-tetrols were as follows: BP-tetrol I-1 (trans-anti-BP-tetrol; 35.2 minutes); BP-tetrol II-1 (trans-syn-BP-tetrol), internal standard (36.9 minutes); and BP-tetrol II-2 (cis-syn-BP-tetrol; 42.3 minutes). Fluorescence was assessed at an excitation wavelength of 344 nm and emission wavelength of 398 nm. As we did not detect the formation of BP-tetrol II-1 in separate analysis of MCF-7 samples, we used it as internal standard (2 pg added to each HPLC run) for verification of the relative retention time. The detection limit was 0.5 pg BP-tetrol I-1 and BP-tetrol II-1. The level of each BP-tetrol was determined by using a standard curve generated from the fluorescence peak area of authentic BP-tetrol standard analyzed just before the analysis of MCF-7 samples. The BP-tetrol I-1 detected is derived after hydrolysis of (+)-anti-BPDE-DNA adduct. The hydrolysis of (−)-anti-BPDE-dG leads to the formation of r-7: t-8/9,10-tetrahydroxy-7,8,9,10-tetrahydro-BP (BP-tetrol II-2), which, however, is unstable and is converted in BP-tetrol I-1 (39). Thus, the level of the formed (−)-anti-BPDE-dG was measured by the quantity of BP-tetrol I-1 found on HPLC runs. Based on the finding that BPDE reacting with DNA produce primarily BPDE-N2-DG (8), we assumed that BP-tetrol I-1 level corresponds to this of BPDE-N2-DG. The level of BPDE bound to MCF-7 DNA was quantified in duplicates. The adduct level was calculated from the equation 1 pmol/mg DNA / 3.125 = 1 adduct per 106 nucleotide. The HPLC runs were quantitatively reproducible, and variability between the two assays was lower than 5%.

Results and Discussions

The mechanism of mutagenesis by BP is sufficiently well defined to be used as a "molecular signature" to establish the causal nature

\[ y = 158.7x - 14.122 \]
\[ R^2 = 0.9966 \]

Figure 2. Results obtained by using a cell-free system concomitant with DNA addition: 6 mg calf thymus DNA in 2 mL water was added to 5 mL CSS with different dilutions and reacted for 2 hours at room temperature with (+)-BP-7,8-diol (final concentration, 3.6 μmol/L). DNA was hydrolyzed and the released BP-tetrol I was measured as described in Materials and Methods.

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Table 1. Effect of scavengers on BPDE-dG level caused by cigarette smoke in a cell-free system

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% BPDE-dG relative to standard cigarette smoke</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard CSS (μg)</td>
<td>100</td>
</tr>
<tr>
<td>-SOD (20)</td>
<td>16</td>
</tr>
<tr>
<td>+Catalase (4)</td>
<td>12</td>
</tr>
<tr>
<td>+Inactivated catalase (4)</td>
<td>100</td>
</tr>
<tr>
<td>Rosemary-filtered CSS instead of standard CSS</td>
<td>42</td>
</tr>
</tbody>
</table>

NOTE: The standard CSS system includes 2 mL calf thymus DNA (3 mg/mL); 600 μL (+)-BP-7,8-diol (30 μmol/L); and 5 mL diluted CSS with PBS (1:19) at pH 7.4 and was incubated at room temperature for 2 hours. Each value was obtained from three independent experiments done in duplicate. The average error was ±12% in each duplicate experiment. The BPDE-dG value of the standard was 56 ± 6.3 (mean ± SD) adducts per milligram DNA.

between particular genetic events in development of tumors and carcinogenic exposure (the “smoking gun”). The “BP molecular signature” has major implication for pinpointing the tobacco smoke as the cause of human lung cancer and for the elaboration of specific strategies to minimize tobacco smoking or introduce preventive measures. Specific agents used in cancer chemoprevention seem to act by inhibiting carcinogen damage to DNA mutagenesis, tumor promotion, and/or tumor progression.

This investigation was designed to investigate the relative role of cigarette smoke on the biotransformation of BP-7,8-diol to BPDE capable of forming stable DNA adduct in human cells. Numerous studies have shown that stable PAH-DNA adducts can lead to mutations through misincorporation of nucleotides or deletion (40). Cigarette smoke is an aerosol of complex chemical composition containing both organic and inorganic compounds, of which 4,800 have been identified thus far (41). Both vapor phase and particulate phase of smoke are known to possess free radicals (27, 42). Whereas the gas-phase radicals are generally short lived, the radicals in the particulate phase are relatively stable and consist of a hydroquinone, semiquinone, quinone complex (25); this complex is an active redox system capable of reducing molecular oxygen to produce superoxide, eventually leading to H2O2 and hydroxyl radicals. In addition, at least 60 different cigarette smoke carcinogens have been implicated in tumor initiation and promotion; the most potent carcinogenic agents contained in cigarette smoke are BP and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (7).

The effect of cigarette smoke on (+)-anti-BPDE-dG using a cell-free system concomitant with DNA adduction. To elucidate the mechanism of BPDE-dG formation dependent from active oxygen generated from cigarette smoke, we looked for this adduct in cell-free in vitro system concomitant with DNA adduction. The CSS solution containing the gas-phase and tar cigarette smoke radicals was immediately reacted with DNA in the presence of (+)-BP-7,8-diol (see protocol above). The results from this experiment show that cigarette smoke can oxidize the (+)-BP-7,8 diol to (+)-anti-BPDE, which in turn form the (-)-anti-BPDE-dG adduct. The amount of (-)-anti-BPDE-dG increased linearly and dose dependently (Fig. 2).

Previously, it was found that large amounts of active oxygen, such as H2O2 and O2−, were generated from cigarette smoke after trapping the smoke in PBS (31). This active oxygen generated from cigarette smoke might be responsible for the observed formation of (-)-anti-BPDE-dG. To verify this, we checked the effect of catalase and superoxide dismutase on the (-)-anti-BPDE-dG produced and found that both enzymes inhibited the formation of the adduct. Inactivated catalase showed no effect (Table 1). From these results, we concluded that cigarette smoke can oxidize (+)-BP-7,8-diol, thus forming (-)-anti-BPDE-dG, and that such capacity can be explained mainly by the action of oxygen generated from cigarette smoke.

The effect of cigarette smoke on (-)-anti-BPDE-dG adduct formed in MCF-7 cells treated with (+)-BP-7,8-diol. Two independent pathways have been shown to participate in the metabolism of BP-7,8-diol to BPDE (Fig. 3; refs. 20–23). The CYP-dependent metabolism of the (+)-enantiomer leads preferentially to (+)-7,8-dihydroxy-c-9,10-oxy-7,8,9,10-tetrahydro-BP, whereas the pathway involving haem-containing proteins in conjunction with a peroxide (e.g., lipid peroxide) preferentially results in (-)-anti-BPDE (20). The (-)-BP-7,8-diol, on the other hand, may be metabolized by both pathways and results in the formation of (+)-anti-BPDE, the ultimate form of BP, and (-)-syn-BPDE (11). The different pathways can be distinguished by HPLC analysis because the tetrots derived from anti-BPDE and syn-BPDE, respectively, are clearly separated under our conditions.

To investigate further the role of cigarette smoke–dependent epoxidation of (+)-BP-7,8-diol leading to the formation of (-)-anti-BPDE that form with DNA (-)-anti-BPDE-dG adduct, human mammary cell line MCF-7 was used. The reason for using MCF-7 cells to see the effect of cigarette smoke ROS on the activation of (+)-BP-7,8-diol was that these cells have little peroxidase activity. The cells were treated with the (+)-BP-7,8-diol, a stereochemical probe that can distinguish the adducts formed by ROS- and CYP-dependent pathways (Fig. 3). Two distinct peaks were observed on the chromatograms corresponding to BP-tetrol I and BP-tetrol II derived from (-)-anti-BPDE-dG and (+)-syn-BPDE-dG, respectively.

Figure 3. A, stereochemistry of BP-7,8-diol epoxidation by peroxyl radicals and CYP to reactive species (anti-BPDE and syn-BPDE) that can bind to DNA. B, acid hydrolysis of DNA to BP-tetrols measured in this study. The hydrolysis of (-)-anti-BPDE-dG and (-)-syn-BPDE leads to the formation of BP-tetrol I-2 and BP-tetrol II-1, which however are unstable and are converted into BP-tetrol I-1 and BP-tetrol II-2.

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Cigarette smoke increased linearly and dose dependently the ROS-dependent formation of (−)-anti-BPDE-dG (Fig. 4A) and decreased the CYP-dependent formation of (+)-syn-BPDE-dG adduct measured by the formation of BP-tetrol II. This decrease is also dose dependant and the inverse of DNA adducts increased linearly with cigarette smoke concentration (Fig. 4B). The inhibitory effect of cigarette smoke on CYP-dependent formation of (+)-syn-BPDE-dG and increased formation of (−)-anti-BPDE-dG adduct confirm the role of the oxygen generated from the cigarette smoke in the formation of (−)-anti-BPDE-dG adduct. Other studies showed that induced CYP activity was impaired by the oxidative challenge. The mechanism underlying such a phenomenon could be a down-regulation of CYP1A1 gene (36–38, 43, 44). Having little peroxidase activity may lead MCF cells under “stress” conditions to increased DNA damage and reduced repair capacity. Consequently, this may cause an increase of BPDE-DNA adducts independently from BP-7,8-diol activation.

**The effect of cigarette smoke on BPDE-dG adduct formed in cells treated with BP.** Previous studies with MCF-7 cell cultures revealed that these cells possess inducible P4501B1 and P4501A1 activity (45). The presence of P450 catalyzed metabolic turnover of BP and the absence of detectable peroxidase activity in MCF-7 cells allowed the evaluation of the role of cigarette smoke oxygen radicals on BP activation in human cell cultures. MCF-7 cells have high CYP1A1 enzyme activity for the metabolic activation of BP, leading to the formation of (−)-BP-7,8-diol and consequently to (+)-anti-BPDE-dG. The level of adduct formation at 6 hours was considerably lower than that observed after 12 and 24 hours of exposure. After treatment with 2.5 μmol/L BP for 6 hours, ~2,000 pg adducts/milligram DNA were formed, whereas >11,000 pg/milligram DNA and >20,000 pg adducts/milligram DNA were present after 12 and 24 hours, respectively (Wilcoxon rank-sum test gives $P = 0.0022$).

The cells were treated for 12 and 18 hours with BP to induce the formation of (−)-BP-7,8-diol, which is substrate for ROS. Indirect confirmation for the preferentially formation of (−)-BP-7,8-diol is the absence of BP-tetrol II derived from syn-BPDE on HPLC runs, which precursor is (+)-BP-7,8-diol. The cells were then exposed for 2 hours with CSS of cigarette smoke together with BP. The HPLC runs show that there is only one peak on chromatograms, which

**Figure 4.** Results obtained using MFC-7 cells. Cells 
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10^6/20 mL were treated for 2 hours with (+)-BP-7,8-diol (0.2 μmol/L) alone or in the presence of different dilutions of CSS. DNA was isolated and hydrolyzed and the released BP-tetrols were measured and the binding levels were determined as described in Materials and Methods. Two distinct peaks were observed on the chromatograms corresponding to BP-tetrol I and BP-tetrol II derived from (−)-anti-BPDE-dG and (+)-syn-BPDE-dG, respectively (32–34). Values are the means plus SD of two independent experiments with three to four HPLC runs. A, top, increases of (−)-anti-BPDE-dG adducts with CSS dilution; B, bottom, increases of 1/(+)-syn-BPDE-dG adduct with CSS dilution.
Cigarette Smoke–Free Radicals and BPDE-DNA Adduct

Figure 5. BPDE-dG binding in MCF-7 cells after exposure to BP (2.5 μmol/L) or BP (2.5 μmol/L) + CSS (dilution for 20 times) for the time indicated. CSS was added on the last 2 hours during the exposure to BP. Analysis of BPDE-dG was done as described in Materials and Methods. Columns, mean of two independent experiments with four to six HPLC runs; bars, SD. The BPDE-dG values were 11.7 ± 0.5 (mean ± SD) µg adducts/milligram DNA after 12 hours of incubation and 17.6 ± 0.4 and 28.1 ± 0.9 after 18 and 24 hours, respectively. The CYP spontaneous metabolism increased theses values to 17.2 ± 0.5, 27.8 ± 0.8, and 42.2 ± 1.0 2 hours after the reference time at 12, 18, and 24 hours, respectively. The addition of CSS induced a much more dramatic change during the same 2-hour period, leading to a final BPDE-dG value of 36.9 ± 1.2, 56.7 ± 0.9, and 80.2 ± 1.2 (mean ± SD) µg adducts/milligram after 12, 18, and 24 hours, respectively.

The effect of filter containing rosemary extract on the formation of BPDE-dG adduct. Rosemary (Rosmarinus officinalis Labiates) herb and oil are commonly used as spice and flavoring agents in food processing for its desirable flavor and high antioxidant activity. Topical application of rosemary extract, carnosol, or ursolic acid to mouse skin inhibited the covalent binding of BP to epidermal DNA, tumor initiation by 7,12-dimethylbenz(a)anthracene, 12-O-tetradecanoylphorbol-13-acetate–induced tumor promotion, ornitine decarboxylase activity, and inflammation (48–51). Rosemary extracts were proved to be efficient not only in the promotion phase (50) but also in the initiation phase (49, 51). Additional studies have revealed that rosemary extracts, carnosic acid, and carnosol strongly inhibited phase I enzyme, CYP activities and induced the expression of the phase II enzyme, GST, and quinone reductase activities (52–54). Carnosol was stated to inhibit nitric oxide production in activated macrophage (55). The antioxidant property had been referred to as the mechanistic basis of their protective effects.

With the aim of removing free radicals and ROS in the cigarette smoke, a small amount of rosemary powder was incorporated in a standard filter (see Materials and Methods). The decrease in free radicals in the condensate induced by the filters incorporating a rosemary extract was estimated by the quantitation of the hydroxyl radical content of CSS with a spin trap (TMPO) using LC-ESI-MS/MS (33). Under the smoking conditions used, a 30% decrease in the hydroxyl radical was observed. Due to the efficiency of this filter to reduce the level of the free radicals in cigarette smoke, compared with a comparable standard Marlboro filter without the additive, we compared the effect of cigarette smoke passed through this filter compared with the standard filter on the formation of BPDE-dG using MCF-7 cells.

The results presented on Fig. 6 were obtained when the MCF-7 cells were treated with BP. Two groups of experiments were done (A and B). The cells were treated with BP for 12 and 18 hours, respectively, following with CSS from the two filters for another 2 hours together with BP. To evaluate the CYP-dependent increase of the adduct during these last 2 hours, two controls for each group were done: 12 and 14 hours for group A as well as 18 and 20 hours for group B. The CSS from the standard filter doubles the binding level obtained for 14 and 20 hours. However, the rosemary filter strongly impedes the increase obtained by the standard filter, >70% in the two groups (Fig. 6). The modified filter scavengers ROS and consequently decreases the activation of (−)-BP-7,8-diol. Aside from the reduction of the free radicals, rosemary powder may have also other mechanisms to reduce BPDE-dG formation. Another study using whole-rosemary extract (6 μg.mL−1) inhibited CYP1A1 activity and DNA adduct formation by 80% after 6 hours of coincubation with 1.5 μmol/L BP in human bronchial epithelial cells (BEAS-2B; ref. 56). Thus, it is possible using the filters that decrease the amount of the free radicals to reduce the formation of the critical tumorigenic adduct, which will be in benefit for the addicted smokers. Our study identifies rosemary cigarette filter as promising candidate for chemopreventive programs with the aim to reduce BPDE-dG adducts in bronchial epithelial cells.

Concluding Remarks

In conclusion, this study shows the following: α) cigarette smoke can activate by its oxygen-generated radicals the second step of BP metabolic way leading to the formation of BPDE-dG adduct,
presumably by metabolism of the formed in the cells (−)-BP-7,8-diol to (+)-anti-BPDE; (b) this activation is at least twice higher than this obtained with CYPs machinery; (c) ROS from the cigarette smoke may be in part responsible for the increased BPDE-dG adduct formation; (d) a filter containing a formulated rosemary powder can reduce considerably the BPDE-dG level due cigarette smoke oxygen-generated radicals; and (e) the methodology in this study can be used for the search of cigarette filters to reduce the formation of carcinogenic BPDE-dG adduct in bronchial epithelial cells.

BP is considered to be important carcinogen involved in lung cancer induction in smokers (7, 10) and, as shown in this study, ROS contribute substantially in the formation of the critical lung tumorigenic adduct. Although it is both critical to prevent addiction to tobacco and to enhance the efficacy of smoking cessation and reduction programs, these approaches have had little effect. The prevention of the formation of BPDE-dG adduct is one approach that may lead to decreasing lung cancer risk in addicted smokers.

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References

Cigarette Smoke–Free Radicals and BPDE-DNA Adduct


DNA Damage by Benzo(a)pyrene in Human Cells Is Increased by Cigarette Smoke and Decreased by a Filter Containing Rosemary Extract, Which Lowers Free Radicals

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