Carcinogenesis vol.18 no.4 pp.795–799, 1997

Benzo[a]pyrene up-regulates cyclooxygenase-2 gene expression in oral epithelial cells

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**Introduction**

Exposure to polycyclic aromatic hydrocarbons is a recognized and important cause of cancer because these otherwise inert chemicals are metabolized by selected enzymes, including P-450s and cyclooxygenase (Cox*, PGH synthase), to carcinogenic and catalyzes prostaglandin biosynthesis. We determined the effects of benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon in tobacco smoke, on cyclooxygenase-2 (Cox-2) mRNA, protein and synthesis of prostaglandin E2 (PGE2) in normal and transformed oral epithelial cells. Treatment with B[a]P caused a dose-dependent increase in production of PGE2, with a maximal increase of ~100%. Enhanced synthesis of PGE2 was associated with increased amounts of Cox-2 protein. B[a]P also caused a two-fold increase in Cox-2 mRNA in both normal and transformed cells. Transient transfections with a Cox-2 promoter construct showed that B[a]P-mediated induction of Cox-2 mRNA reflected increased transcription. Levels of Cox-1 were unaffected by B[a]P. B[e]P did not affect the synthesis of PGE2 or amounts of Cox-2. These data are important because B[a]P-mediated induction of Cox-2 may predispose to carcinogenesis by enhancing the production of mutagens and the synthesis of prostaglandins.

*Abbreviations: Cox, cyclooxygenase-2; PGE2, prostaglandin E2; B[a]P, benzo[a]pyrene; B[e]P, benzo[e]pyrene; FBS, fetal bovine serum; LDH, lactate dehydrogenase; NSAI.D, non-steroidal anti-inflammatory drug; SDS, sodium dodecyl sulfate; SSPE, saline–sodium phosphate–EDTA buffer; XRE, xenobiotic responsive element.

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PGE$_2$ by enzyme immunoassay. Values are means for 6 h. Culture medium was removed and then assayed for material. Protein concentration was measured by the method of Lowry.

Fig. 1. Benzo[a]pyrene induces the synthesis of PGE$_2$ by oral epithelial cells. 1483 cells were treated with vehicle (0.01% DMSO) or B[a]P (125–1000 ng/ml) for 4.5 h. Cellular lysate protein (100 µg/lane) was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The nitrocellulose membrane was probed with antibody specific for Cox-2. Results of densitometry in arbitrary units were as follows: lane 2, 23 ± 5; lane 3, 41 ± 17; lane 4, 57 ± 13; lane 5, 58 ± 6; lane 6, 71 ± 6. Values are means ± SD; n = 3. *P = 0.01 compared with control, **P < 0.01 compared with control.

Fig. 2. Benzo[a]pyrene induces Cox-2 in oral epithelial cells. 1483 cells were treated with vehicle (0.01% DMSO, lane 2) or B[a]P (125–250, 500, 1000 ng/ml; lanes 3–6) for 4.5 h. Lane 1 represents ovine Cox-2 which was used as a standard. Cellular lysate protein (25 µg/lane) was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The nitrocellulose membrane was probed with antibody specific for Cox-2. The protein concentration was measured by the method of Lowry.

Fig. 3. Benzo[a]pyrene does not induce Cox-2 in oral epithelial cells. 1483 cells were treated with vehicle (0.01% DMSO, lane 2), B[a]P (1000 ng/ml, lane 3) or B[a]P (1000 ng/ml; lane 4) for 4.5 h. Lane 1 represents ovine Cox-2, which was used as a standard. A 25 µg aliquot of cellular lysate protein was used in each lane. The nitrocellulose membrane was probed with anti-Cox-2 antisera. Results of densitometry in arbitrary units (a.u.) were as follows: lane 2, 44 a.u.; lane 3, 102 a.u.; lane 4, 46 a.u.

Cellular cytotoxicity was assessed by release of LDH and trypan blue exclusion. Levels of LDH were measured in the supernatants used for PGE$_2$ analyses. LDH assays were performed according to the manufacturer’s instructions. For trypan blue analysis, following treatment with B[a]P for 3–6 h, cells were trypsinized and combined 1:1 with 0.4% trypan blue and examined for dye exclusion.

PGE$_2$ Production

Cells were plated at a density of 2.5×10$^5$ cells/well in 6-well plates and allowed to attach for 24 h prior to experiments. Medium was obtained from each well and centrifuged to sediment cell debris; the supernatants were frozen at −80°C until assay. Supernatants were assayed for spontaneously released PGE$_2$ by enzyme immunoassay (15). To normalize the PGE$_2$ data, cell number was measured with an electronic particle counter (Coulter, Hialeah, FL).

Fig. 4. Benzo[a]pyrene does not affect amounts of Cox-1. 1483 cells were treated with vehicle (0.01% DMSO, lane 1) or B[a]P (1000 ng/ml, lane 2) for 4.5 h. Cellular lysate protein (100 µg/lane) was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody specific for Cox-1. Densitometry was performed and the results expressed in arbitrary units (a.u.): lane 1, 533 a.u.; lane 2, 491 a.u.

Western blotting

Cells were plated in 100-mm Petri dishes at 1.5–2.0×10$^5$ cells/dish and allowed to attach for 24 h prior to experiments. Cell lysates were prepared by treating cells with lysis buffer consisting of 150 mM NaCl, 100 mM Tris-buffered saline, 1% Tween-20, 50 mM diethylthioldiacarbamate, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Lysates were sonicated twice for 20 s and centrifuged at 10 000 g for 10 min to sediment the particulate material. Protein concentration was measured by the method of Lowry et al. (16) and the supernatant stored at −80°C until assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (17). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (18). The nitrocellulose membrane was then incubated with a rabbit polyclonal antibody raised against the unique 18-amino acid sequence from the carboxy-terminal portion of Cox-2 that does not react with Cox-1 (19). Nitrocellulose membranes were also probed with a monoclonal anti-Cox-1 antibody (19). The membrane was subsequently probed with a goat anti-rabbit or a goat anti-mouse antibody conjugated to alkaline phosphatase as described previously (20).

A computer densitometer (Molecular Dynamics, Sunnyvale, CA) was used to determine the density of the bands.

Northern blotting

Cells were plated in 100-mm Petri dishes at a density of 1.5–2.0×10$^5$ cells/dish and allowed to attach for 24 h prior to experiments. To prepare total cellular RNA, cell monolayers were washed and then directly lysed in 4 mol/l guanidinium isothiocyanate solution. RNA was then isolated by phenol–chloroform extraction according to Chomczynski and Sacchi (21). For Northern blots, 15 µg of total cellular RNA per lane was electrophoresed in formaldehyde–

hydro-containing agarose gels and transferred to nylon-supported membranes. After baking, membranes were prehybridized for 3 h and then hybridized in a solution containing 50% formamide, 5× saline–sodium phosphate–EDTA buffer (SSPE), 5% Denhardt’s solution, 0.1% SDS, and 100 µg/ml single-stranded salmon sperm DNA. Hybridization was carried out for 16 h at 42°C with a human Cox-2 cDNA probe. To verify the identity of RNA loading in the different lanes, the blot was stripped of radioactivity and rehybridized to determine levels of 18S rRNA. Cox-2 and 18S rRNA probes were labeled with [32P]CTP by random priming. After hybridization, membranes were washed for 20 min at room temperature in 2× SSPE-0.1% SDS, twice for 20 min in the same solution at 55°C, and twice for 20 min in 0.1× SSPE-0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography. The signal level of the bands was quantified by densitometry. Transient transfection assays

1483 cells were seeded at a density of 8×10$^5$ cells/well in 6-well dishes and grown to 30–40% confluence in DMEM/F-12 containing 10% FBS. For each well, 2 µg of plasmid DNA containing 1432 bases of 5′-flanking region of Cox-2 ligated to luciferase, was transfected (22). Transfections were done with pFx-3 at a 1:12 ratio of DNA to lipid in Opti-MEM for 12 h as per the manufacturer’s instructions. Subsequently, cells were treated with medium containing different concentrations of B[a]P (0–1000 ng/ml). Luciferase activity was measured in cellular extract 24 h later.

Luciferase activity was measured as follows. Each well was washed twice with PBS. 200 µl of 1× lysis buffer (Analytical Luminescence Laboratories, San Diego, CA) was added to each well for 30 min. Lysate was centrifuged for 5 min at 4°C. The supernatant was used to assay luciferase activity and the protein concentration. Luciferase activity was measured using a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, San Diego, CA) according to the manufacturer’s instructions. Luciferase activities were normalized to protein concentrations.

Statistics

Comparisons between groups were made by the Student’s t-test. A difference between groups of P < 0.05 was considered significant.
significant increase in the production of PGE 2 (Table I).

Table I. Benzo[a]pyrene does not enhance production of PGE2 by oral epithelial cells

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Production of PGE2 (pg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.019 ± 0.003</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>0.036 ± 0.005*</td>
</tr>
<tr>
<td>Benzo[e]pyrene</td>
<td>0.025 ± 0.003</td>
</tr>
<tr>
<td>7,12-Dimethylbenz[a]anthracene</td>
<td>0.023 ± 0.005</td>
</tr>
</tbody>
</table>

1483 cells were treated with vehicle (0.01% DMSO) or 1000 ng/ml B[a]P. B[e]P or 7,12-dimethylbenz[a]anthracene for 6 h. Culture medium was removed and assayed for PGE2 by enzyme immunoassay. Values are means ± SD; n = 6. *P < 0.01.

**Results**

**Effect of B[a]P on PGE2 synthesis**

Induction of Cox by B[a]P can be assessed in a variety of ways including changes in the synthesis of PGE2. Using this assay, B[a]P caused a dose-dependent increase in synthesis of PGE2 in transformed oral epithelial cells (Figure 1). Treatment with B[a]P at a dose of 1000 ng/ml resulted in about an 80% increase in production of PGE2; higher doses of B[a]P did not cause further increases in prostaglandin synthesis. Similar effects of B[a]P were observed after treatment of cells for 6 or 12 h. Normal oral epithelial cells also were used to investigate the effect of B[a]P on synthesis of PGE2. Treatment with B[a]P (1000 ng/ml) led to approximately a 100% increase in PGE2 synthesis in these cells; higher concentrations of B[a]P did not cause further increases in synthesis of PGE2 (data not shown). In contrast to the inducing effects of B[a]P, B[e]P and 7,12-dimethylbenz[a]anthracene did not cause a significant increase in the production of PGE2 (Table I).

Measurements of prostaglandin production were done when cells were equally confluent because differences in cell proliferation affect the release of arachidonic acid (23). In these experiments, cytotoxicity was assessed by cell number, LDH release and trypan blue exclusion. No evidence of cellular toxicity was detected (data not shown).

**B[a]P induces Cox-2 in oral epithelial cells**

The possibility that the above differences in production of PGE2 reflected at least, in part, differences in levels of Cox was evaluated by Western blotting of cell lysate protein. As shown in Figure 2, B[a]P caused a dose-dependent increase in amounts of Cox-2. In contrast to the inducing effects of B[a]P, B[e]P had no effect on levels of Cox-2 (Figure 3), consistent with the inability of this compound to enhance the production of PGE2. Levels of Cox-1 were unaffected by B[a]P (Figure 4).

Since B[a]P-mediated induction of Cox-2 enzyme could reflect either increased protein synthesis or decreased degradation, Northern blotting of Cox-2 mRNA was carried out. In representative experiments shown in Figure 5, higher levels of Cox-2 mRNA were detected in normal and transformed cells after treatment with B[a]P. In fact, B[a]P caused a dose-dependent increase in levels of Cox-2 mRNA. B[a]P at a dose of 1000 ng/ml led to about a two-fold increase in amounts of Cox-2 mRNA in both types of cells.

Differences in levels of mRNA could reflect altered rates of transcription or mRNA stability. To distinguish between these two possibilities, transient transfections were conducted using a human Cox-2 luciferase reporter construct. As shown in Figure 6, B[a]P caused a doubling of luciferase activity consistent with increased transcription.

**Discussion**

In this study, we showed that B[a]P induced Cox-2 in oral epithelial cells. This observation is important for a variety of
reasons. To begin with, in extrathoracic tissues such as those of the head and neck in which P450 content is low (24), Cox is likely to be important for carcinogen metabolism. Cox converts a broad array of carcinogens to reactive metabolites, which can form DNA adducts (4–8). Thus, several classes of chemical carcinogens, e.g. aromatic amines, heterocyclic amines and dihydrodiol derivatives of polycyclic aromatic hydrocarbons, are activated to mutagenic derivatives by Cox. It is possible, therefore, that B[a]P-mediated induction of Cox-2 will amplify the effect of a given dose of B[a]P on tumor initiation. The potential importance of this idea is underscored by the recent report that B[a]P-diolepoxide, a mutagen formed by Cox, causes adducts along exons of the p53 gene that correspond to p53 mutational hot spots in human lung cancer (25). Another interesting possibility is that up-regulation of Cox-2 will enhance the activation of tobacco-specific nitrosoamines such as NNK or NNK (26). Aside from potentially activating NF-κB and various other mechanisms, the oxidative function of Cox produces prostaglandins that mediate inflammation, that down-regulate mechanisms responsible for B[a]P-mediated activation of 7,12-dihydro-7,12-dihydroxy-benz(a)anthracene stimulated transcription of the Cox-2 gene via an XRE-independent mechanism suggests that the Ah receptor may not be involved in mediating the effects of B[a]P (42). B[a]P-induced oxidative stress could activate NF-kB or AP-1 (43) and thereby up-regulate Cox-2. This idea is attractive because it fits with the observation that antioxidants protect against B[a]P-induced cellular transformation (44). Further studies are needed to elucidate the molecular mechanisms responsible for B[a]P-mediated up-regulation of Cox-2.

Acknowledgements

The authors wish to thank Dr David Zakim for suggestions that were helpful in preparing this manuscript. This work was supported by grants CA68136, CA57166 and 1T32 CA09685-02 from the National Cancer Institute. J.R.M. was the recipient of a fellowship award from the Cancer Research Foundation of America.

References


Received on September 19, 1996; accepted on December 6, 1996

B[et]p upregulation of cyclo-oxygenase gene expression