

# Levels of Cyclooxygenase-2 Are Increased in the Oral Mucosa of Smokers: Evidence for the Role of Epidermal Growth Factor Receptor and Its Ligands

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## Abstract

Cyclooxygenase-2 (COX-2) is a promising pharmacologic target for preventing aerodigestive malignancies. In this study, we investigated the effects of tobacco smoke on the expression of COX-2 in oral mucosa. An ~4-fold increase in amount of COX-2 mRNA was observed in the oral mucosa of active smokers versus never smokers. Thus, a series of *in vitro* studies were carried out to elucidate the mechanism by which tobacco smoke induced COX-2. Treatment of a nontumorigenic oral epithelial cell line (MSK-Leuk1) with a saline extract of tobacco smoke (TS) stimulated COX-2 transcription, resulting in increased amounts of COX-2 mRNA, COX-2 protein, and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis. Exposure of cells to TS also caused an increase in epidermal growth factor receptor (EGFR) tyrosine kinase activity. Both an inhibitor of EGFR tyrosine kinase activity and a neutralizing anti-EGFR antibody blocked TS-mediated induction of COX-2. To define the mechanism by which TS activated EGFR, the release of amphiregulin and transforming growth factor  $\alpha$ , two ligands of the EGFR, was measured. Exposure to TS caused a rapid increase in the release of both ligands. TS also markedly induced the expression of mRNAs for amphiregulin and transforming growth factor  $\alpha$ . Importantly, increased expression of both ligands was also detected in the oral mucosa of active smokers. Taken together, these results suggest that activation of EGFR signaling contributes to the elevated levels of COX-2 found in the oral mucosa of smokers. Moreover, these findings strengthen the rationale for determining whether inhibitors of COX-2 or EGFR tyrosine kinase activity can reduce the risk of tobacco smoke-related malignancies of the aerodigestive tract. (Cancer Res 2005; 65(2): 664-70)

## Introduction

Cyclooxygenases (COX) catalyze the first step in synthesis of prostaglandins (PG) from arachidonic acid. There are two isoforms of COX. One is constitutive (COX-1), and the other is inducible (COX-2; ref. 1). Separate but related genes encode COX-1 and COX-2. The COX-2 gene is an immediate, early-response gene that is induced by a variety of mitogenic and inflammatory

stimuli (1–5). The constitutive isoform, COX-1, is generally unaffected by these factors (1).

Multiple lines of evidence suggest that COX-2 has a significant role in carcinogenesis. COX-2 is the isoform that is overexpressed in transformed cells (3) and in premalignant and malignant tissues (5–10). The most specific data that support a cause-and-effect relationship between COX-2 and tumorigenesis come from genetic studies. Overexpression of COX-2 in the mammary glands of transgenic mice resulted in enhanced tumorigenesis (11). In a related study, transgenic mice that overexpressed COX-2 in skin developed epidermal hyperplasia and dysplasia (12). Conversely, mice engineered to be COX-2 deficient are protected against developing both skin and intestinal tumors (13, 14). In addition to the genetic evidence, numerous pharmacologic studies suggest that COX-2 is a *bona fide* therapeutic target. Selective COX-2 inhibitors protect against the formation and growth of experimental tumors including those of the aerodigestive tract (15–22). Moreover, treatment with celecoxib, a selective COX-2 inhibitor, reduced the colorectal polyp burden in patients with familial adenomatous polyposis (23). Several potential mechanisms may explain the link between COX-2 and malignancy. Enhanced synthesis of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which occurs in neoplastic tissues, has been shown to stimulate cell proliferation (24, 25), induce angiogenesis (26, 27), inhibit apoptosis (28) and suppress immune surveillance (29, 30). Importantly, COX-2 can also catalyze the synthesis of DNA-damaging compounds including those found in tobacco smoke (31, 32).

Smoking is an important risk factor for multiple human malignancies including cancers of the aerodigestive tract (33). Although cessation of tobacco use is highly desirable, it is not realistic for everyone. Hence, there is a significant need for chemopreventive agents that protect against the carcinogenic effects of tobacco smoke. Treatment with  $\beta$ -carotene increased the risk of lung cancer in smokers underscoring the need for new therapeutic strategies (34).

In the present study, we first determined that COX-2 was overexpressed in the oral mucosa of active smokers versus never smokers and then attempted to elucidate the underlying mechanism. The results of *in vitro* studies indicated that tobacco smoke stimulated epidermal growth factor receptor (EGFR) tyrosine kinase activity leading, in turn, to enhanced transcription of COX-2. Because both COX-2 and EGFR have been implicated in carcinogenesis, these findings highlight the potential importance of COX-2 and EGFR as molecular targets for the chemoprevention of tobacco smoke-related malignancies of the aerodigestive tract.

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## Materials and Methods

**Materials.** DMEM:Ham's F-12 medium and fetal bovine serum were from Life Technologies, Inc. (Grand Island, NY). Keratinocyte basal and growth media were from Clonetics Corp. (San Diego, CA). Sodium arachidonate and kits for lactate dehydrogenase and Lowry protein measurements were from Sigma Chemical Co. (St. Louis, MO). Enzyme immunoassay reagents for PGE<sub>2</sub> assays and COX-2 cDNA were from Cayman Chemical Co. (Ann Arbor, MI). Complete mini protease inhibitor mixture was from Roche Diagnostics (Mannheim, Germany). Enzyme immunoassay kits for amphiregulin and TGF- $\alpha$  were purchased from R&D Systems, Inc. (Minneapolis, MN) and Oncogene Research Products (San Diego, CA), respectively. Amphiregulin and TGF- $\alpha$  were obtained from R&D Systems, Inc. [ $\alpha$ -<sup>32</sup>P]dCTP was from Perkin-Elmer Life Sciences (Boston, MA). Random-priming kits were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). RNA was prepared using kits from Qiagen (Chatsworth, CA). The 18S rRNA cDNA was from Ambion, Inc. (Austin, TX). Anti-human COX-2 polyclonal antiserum and anti-phosphotyrosine antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to EGFR were purchased from Upstate (Lake Placid, NY). Western blotting detection reagents were from Amersham Biosciences (Piscataway, NJ). AG1478 was purchased from Calbiochem (La Jolla, CA).

**Human Tissue.** Buccal mucosa specimens were obtained from 9 never smokers and 10 active smokers ( $\geq 10$  pack-years). Subjects were excluded if they had gross evidence of oral inflammation, a history of heavy alcohol consumption, or recent use of nonsteroidal anti-inflammatory drugs or other anti-inflammatory medications. After topical anesthesia, 3-mm punch biopsies were obtained from grossly normal appearing buccal mucosa. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. This study was approved by the Committee on Human Rights in Research at Weill Medical College of Cornell University.

**Tissue Culture.** The MSK-Leuk1 cell line was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue (35). Cells were routinely maintained in keratinocyte growth medium supplemented with bovine pituitary extract. Human 1483 squamous carcinoma cells have been described previously (36). The non-small cell lung cancer cell line H2122 was a generous gift of Dr. Raphael A. Nemenoff (University of Colorado, Denver, CO; ref. 37). Cells were grown in basal medium for 24 hours before treatment. Treatment with vehicle (PBS) or a saline extract of tobacco smoke (TS, see below) was carried out under serum-free conditions. Cellular cytotoxicity was assessed by measurements of cell number, release of lactate dehydrogenase and trypan blue exclusion. There was no evidence of cytotoxicity in any of the experiments.

**Preparation of Tobacco Smoke.** Cigarettes (Marlboro, King Size) were smoked in a Borgwaldt piston-controlled apparatus (Model RG-1, Hamburg, Germany) using the Federal Trade Commission standard protocol. The protocol parameters attempt to mimic a standardized human smoking pattern (duration, 2 seconds/puff; frequency, 1 puff/min; volume, 35 mL/puff). Cigarettes were smoked one at a time in the apparatus and the smoke drawn under sterile conditions into premeasured amounts of sterile PBS, pH 7.4. This smoke in PBS represents whole trapped mainstream smoke, abbreviated as TS. Quantitation of smoke content is expressed in puffs/mL of PBS with one cigarette yielding about 8 puffs drawn into a 5 mL volume. The final concentration of TS in the cell culture medium is expressed as puffs per mL medium.

**PGE<sub>2</sub> Production.** Cells ( $5 \times 10^4$  per well) were plated in six-well dishes and grown to 60% confluence in growth medium. At the end of the treatment period, the medium was replaced with fresh medium containing 10  $\mu\text{mol/L}$  sodium arachidonate. Thirty minutes later, the medium was collected for analysis of PGE<sub>2</sub>. Production of PGE<sub>2</sub> was determined by enzyme immunoassay according to the manufacturer's instructions. Amounts of PGE<sub>2</sub> are expressed as picograms per microgram cellular protein.

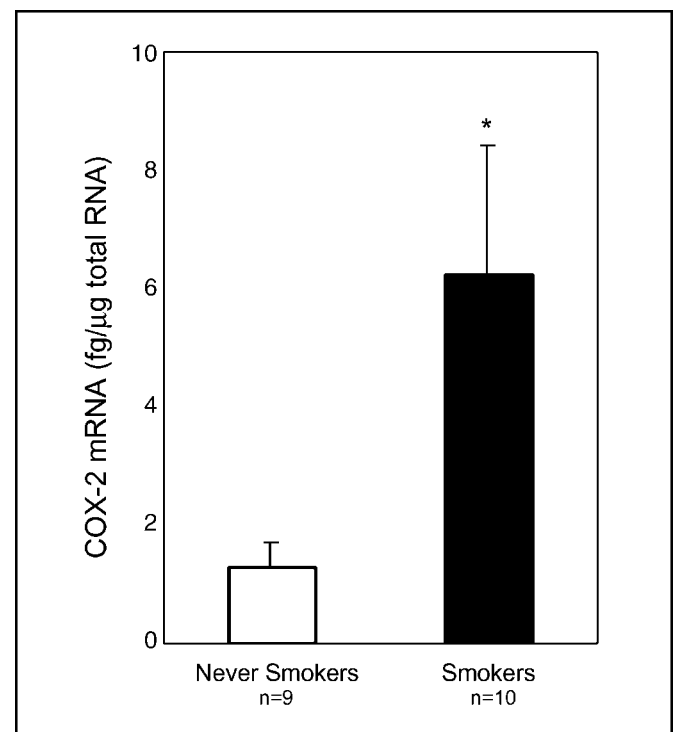
**Immunoprecipitation and Western Blotting.** For EGFR, immunoprecipitates were prepared by treating cells with lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 50 mmol/L NaF, 30 mmol/L sodium

pyrophosphate, 5 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 5  $\mu\text{g/mL}$  aprotinin, Complete mini protease inhibitor mixture, and 5  $\mu\text{mol/L}$  3,4 dichlorocoumarin]. Lysates were sonicated for 20 seconds on ice and centrifuged at  $10,000 \times g$  for 10 minutes to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (38). The supernatant was preabsorbed with 20  $\mu\text{L}$  of normal goat immunoglobulin G at  $4^{\circ}\text{C}$ ; 80  $\mu\text{L}$  of protein G PLUS-agarose was then added. The mixture was then centrifuged at  $3,000 \times g$  for 5 minutes at  $4^{\circ}\text{C}$ . The pellet was discarded. Four microliters of monoclonal anti-human EGFR antiserum was added to the supernatant; the mixture was then incubated at  $4^{\circ}\text{C}$  on a rocker platform overnight. Eighty microliters of protein G PLUS-agarose was then added, and the mixture was then incubated at  $4^{\circ}\text{C}$  on a rocker platform for 2 hours; the mixture was then centrifuged at  $3,000 \times g$  for 5 minutes at  $4^{\circ}\text{C}$ . The supernatant was discarded. After washing the pellet four times with lysis buffer, the pellet was resuspended. Cell lysates for COX-2 analysis were prepared as previously described (39).

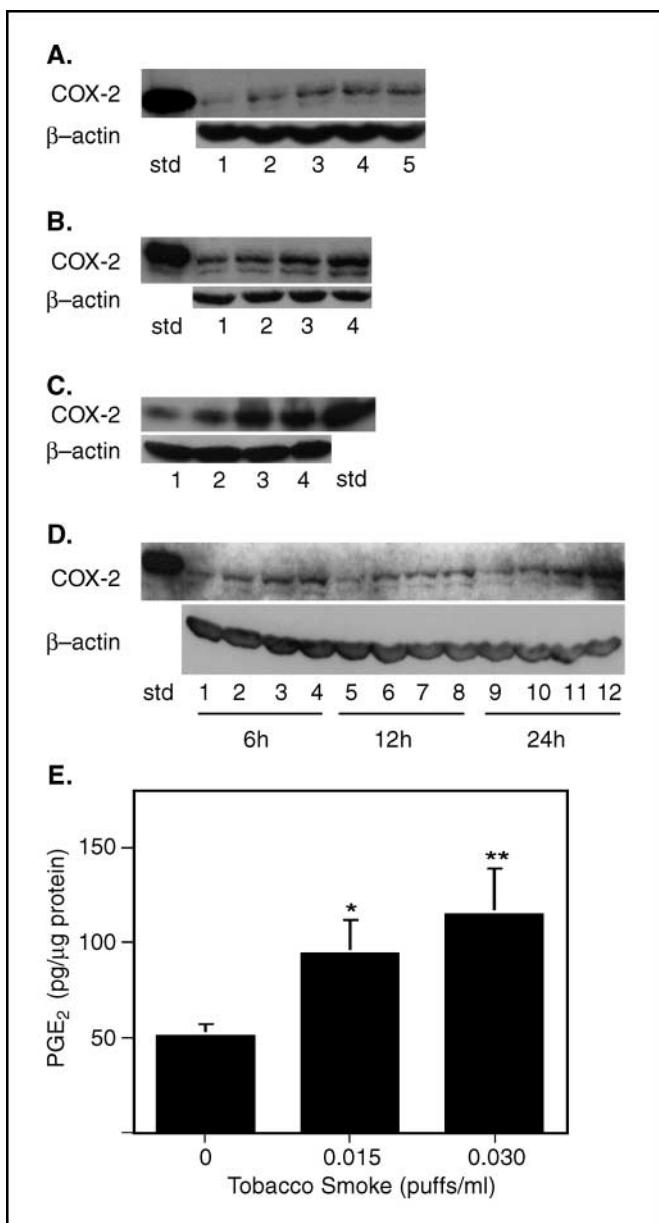
SDS-PAGE was then done under reducing conditions on 7.5% or 10% polyacrylamide gels. The resolved proteins were transferred onto nitrocellulose sheets. The nitrocellulose membrane was then incubated with primary antisera to phosphotyrosine, EGFR, COX-2, or  $\beta$ -actin. Secondary antibody to immunoglobulin G conjugated to horseradish peroxidase was used. The blots were then reacted with enhanced chemiluminescence Western blot detection system according to the manufacturer's instructions.

**Northern Blotting.** Total cellular RNA was isolated from cell monolayers using the RNA isolation kit from Qiagen. Ten micrograms of RNA per lane were electrophoresed in a formaldehyde-containing 1% agarose gel and transferred to nylon-supported membranes. The membrane was hybridized with radiolabeled cDNA probes for human COX-2 and 18S rRNA, respectively, as described previously (3, 5, 39).

**Transient Transfection Assays.** Cells were seeded at a density of  $5 \times 10^4$  cells/well in six-well dishes and grown to 50% to 60% confluence.



**Figure 1.** Increased levels of COX-2 are detected in the buccal mucosa of smokers. Biopsies of buccal mucosa were obtained from 9 never smokers and 10 active smokers ( $\geq 10$  pack-years). Quantitative RT-PCR was used to determine the amounts of COX-2 mRNA in these samples. Columns, means; bars, SD. \*,  $P < 0.05$ .



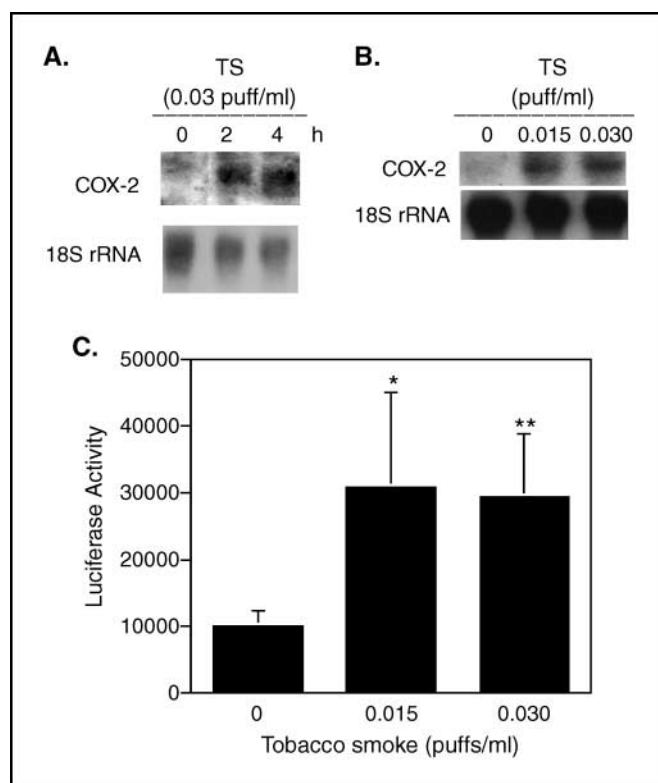
**Figure 2.** Tobacco smoke induces COX-2 protein and PGE<sub>2</sub> synthesis in cell lines derived from the human aerodigestive tract. MSK-Leuk1 (A and E), 1483 cells (B and D), and H2122 (C) cells were treated with TS for 6 to 24 hours. A, lysate protein was from MSK-Leuk1 cells treated with vehicle (lane 1), TS (0.0075 puffs/mL, lane 2), TS (0.015 puffs/mL, lane 3), TS (0.030 puffs/mL, lane 4) or TS (0.06 puffs/mL) for 12 hours. B, lysate protein was from 1483 cells treated with vehicle (lane 1), TS (0.0075 puffs/mL, lane 2), TS (0.015 puffs/mL, lane 3), or TS (0.030 puffs/mL, lane 4) for 24 hours. C, lysate protein was from H2122 cells treated with vehicle (lane 1), TS (0.0075 puffs/mL, lane 2), TS (0.015 puffs/mL, lane 3), or TS (0.030 puffs/mL, lane 4) for 24 hours. D, lysate protein was from 1483 cells treated with vehicle (lanes 1, 5, and 9), TS (0.0075 puffs/mL, lanes 2, 6, and 10), TS (0.015 puffs/mL, lanes 3, 7, and 11), or TS (0.030 puffs/mL, lanes 4, 8, and 12) for 6 hours (lanes 1-4), 12 hours (lanes 5-8), and 24 hours (lanes 9-12), respectively. In A to D, cellular lysate protein (100  $\mu$ g/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. Immunoblots were probed sequentially for COX-2 and  $\beta$ -actin. E, MSK-Leuk1 cells were treated with vehicle or the indicated concentration of TS for 12 hours. The medium was then replaced with fresh basal medium and 10  $\mu$ M sodium arachidonate. Thirty minutes later, the medium was collected to determine the synthesis of PGE<sub>2</sub>. Production of PGE<sub>2</sub> was determined by enzyme immunoassay. Columns, means ( $n = 6$ ); bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

The -1432/+59 COX-2 promoter-luciferase construct used for transfections was a generous gift of Dr. Tadashi Tanabe (National Cardiovascular Research Institute, Osaka, Japan; refs. 39, 40). For the cells in each well, 1  $\mu$ g of plasmid DNA was introduced using 2  $\mu$ g of LipofectAMINE 2000 according to the manufacturer's instructions. After 12 hours of incubation, the medium was replaced with basal medium. The activities of luciferase and  $\beta$ -galactosidase were measured in cellular extracts as in previous studies (39, 41).

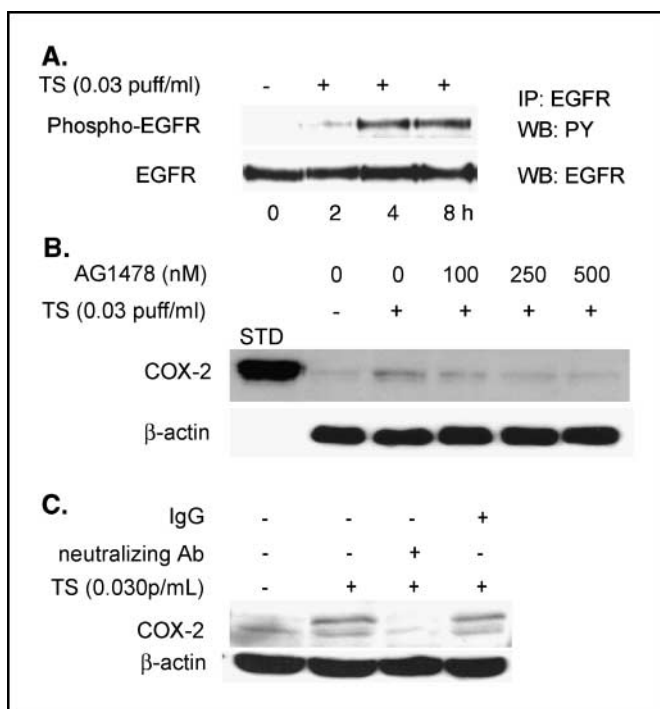
**Quantitative PCR for COX-2.** Total RNA was isolated from buccal mucosa samples using RNeasy mini kits from Qiagen. Total RNA was reverse transcribed using the GeneAmp RNA PCR kit according to the manufacturer's instructions. Quantitative PCR for COX-2 was carried out as described previously (8).

**Measurements of Ligands of EGFR.** MSK-Leuk1 cells were plated in six-well dishes and grown to 60% confluence in growth medium. Following treatment, levels of amphiregulin and TGF- $\alpha$  released in the medium were quantified by enzyme immunoassay according to the manufacturer's instructions. Amounts of ligand in the medium are expressed as picograms per microgram cellular protein.

To determine cellular levels of mRNAs for amphiregulin, TGF- $\alpha$ , and HB-EGF, total cellular RNA was isolated from cells according to the manufacturer's instructions. Reverse transcription was done in a thermocycler using 2  $\mu$ g of RNA per 40  $\mu$ L of reaction. The reaction mixture contained 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L deoxynucleotide triphosphate, 2.5  $\mu$ mol/L oligo(dT)<sub>16</sub> primer, 40 units RNase inhibitor, and 100 units murine leukemia virus reverse transcriptase (Roche Applied Science, Indianapolis, IN). Samples were amplified for 10 minutes at



**Figure 3.** Tobacco smoke induces COX-2 mRNA and stimulates COX-2 promoter activity. A, MSK-Leuk1 cells were treated with vehicle or TS (0.030 puffs/mL) for 2 or 4 hours. B, 1483 cells were treated with vehicle (lane 1) or the indicated concentration of TS for 4 hours. Total cellular RNA was isolated; 10  $\mu$ g of RNA was added to each lane. The Northern blot was probed sequentially for COX-2 mRNA and 18S rRNA. C, MSK-Leuk1 cells were transfected with the -1432/+59 COX-2 promoter. After transfection, cells were treated with vehicle or the indicated concentration of TS. Reporter activities were measured in cellular extracts 12 hours after treatment. Luciferase activity represents data that have been normalized to  $\beta$ -galactosidase activity. Columns, means ( $n = 6$ ); bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with vehicle-treated control.



**Figure 4.** Tobacco smoke induces COX-2 by an EGFR-dependent mechanism. **A**, MSK-Leuk1 cells were treated with vehicle or TS (0.030 puffs/mL) for up to 8 hours. Cell lysates were immunoprecipitated (IP) with anti-EGFR antibody, immunoblotted (WB) with anti-phosphotyrosine antibody (PY, top) or anti-EGFR antibody (bottom), and visualized with chemiluminescence. **B**, MSK-Leuk1 cells were pretreated with vehicle or the indicated concentration of AG1478 for 2 hours. Subsequently, cells were treated with vehicle, TS (0.030 puffs/mL), or TS plus the indicated concentration of AG1478 for 12 hours. **C**, MSK-Leuk1 cells were pretreated for 2 hours with vehicle, EGFR-neutralizing antibody (10  $\mu$ g/mL), or mouse immunoglobulin G (10  $\mu$ g/mL). Subsequently, cells were treated with vehicle or TS (0.030 puffs/mL) for 12 hours. In **B** and **C**, cellular lysate protein (100  $\mu$ g/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed sequentially for COX-2 and  $\beta$ -actin.

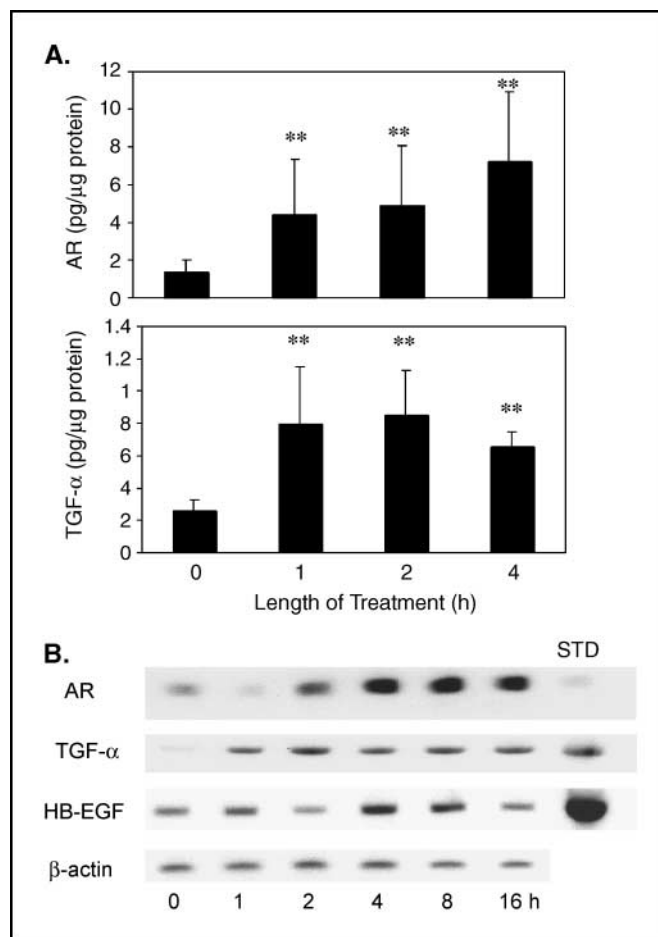
25°C, 15 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 5°C. The resulting cDNA was then used for amplification. The volume of the PCR reaction was 25  $\mu$ L and contained 2  $\mu$ L cDNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L MgCl<sub>2</sub>, 0.4 mmol/L deoxynucleotide triphosphates, 400 nmol/L forward primer, 400 nmol/L reverse primer, and 2.5 units Taq polymerase (Applied Biosystems, Foster City, CA). Samples were denatured at 95°C for 2 minutes and then amplified for 35 cycles in a thermocycler under the following conditions: 95°C for 30 seconds, 62°C for 30 seconds, and then 70°C for 45 seconds. Subsequently, extension was carried out at 70°C for 10 minutes. Primer sequences were as follows: amphiregulin, forward 5'-AAGCGTGAACCATTTCTGG-3', reverse 5'-TTTCATGGACTTTTCCCCAC-3'; TGF- $\alpha$ , forward 5'-GTGTGCAG-CAGTTGGTGTCT-3', reverse 5'-CCCAAGCCTTAGCTGTCTT-3'; HB-EGF, forward 5'-GGTGGTGTGAAGCTTTC-3', reverse 5'-CCTAAACATGA-GAAGCCCA-3'; and  $\beta$ -actin, forward 5'-GGTACCCACACTGTGCCAT-3', reverse 5'-GGATGCCACAGACTCCATGC-3'. To determine levels of mRNAs for amphiregulin and TGF- $\alpha$  in buccal mucosa, total RNA was isolated from biopsy samples using RNeasy mini kits from Qiagen. Analysis was carried out as described above. Importantly, the signal for each product was linear with the amount of RNA analyzed. PCR products were electrophoresed on a 1% agarose gel with 0.5  $\mu$ g/mL ethidium bromide and photographed under UV light. The identity of each PCR product was confirmed by DNA sequencing. A computer densitometer (Chem Doc; Bio-Rad, Hercules, CA) was used to quantify the density of the different bands.

**Statistics.** Comparisons between groups were made by the Student's *t* test. A difference between groups of *P* < 0.05 was considered significant.

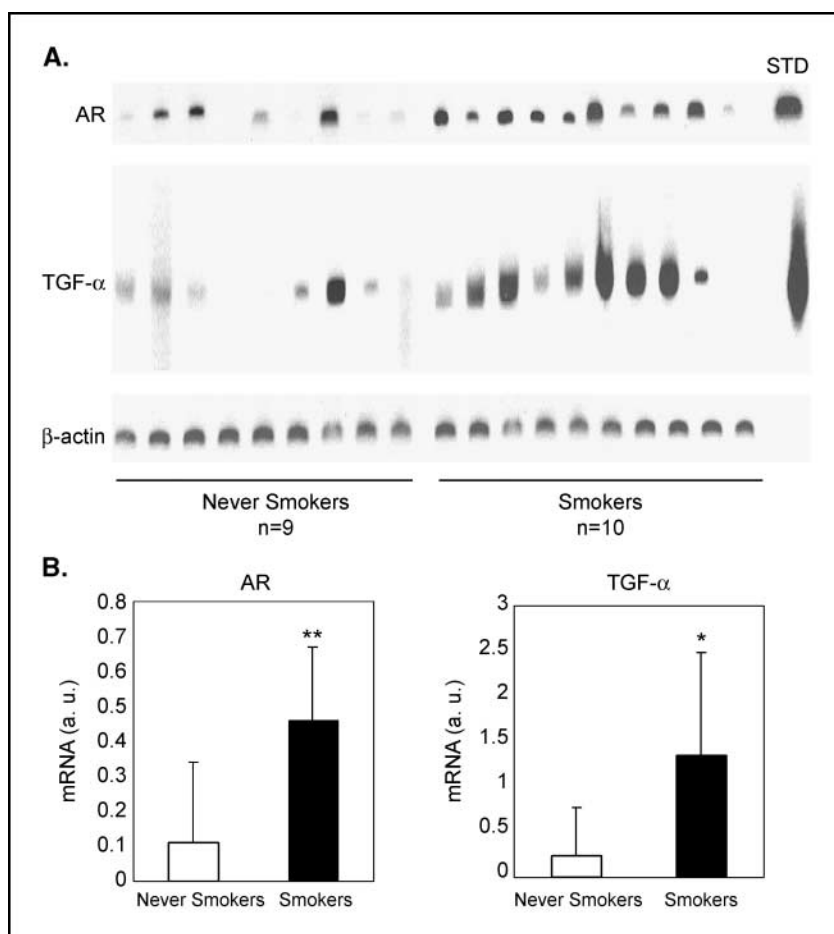
## Results

Quantitative reverse transcription-PCR (RT-PCR) was carried out to determine amounts of COX-2 mRNA in the buccal mucosa of smokers (*n* = 10) versus never smokers (*n* = 9). A nearly 4-fold increase in mean levels of COX-2 message was detected in smokers (Fig. 1). To determine whether this might reflect a direct effect of tobacco smoke, *in vitro* studies were carried out. Treatment with TS caused dose-dependent induction of COX-2 protein in a nontumorigenic cell line derived from a premalignant oral lesion (MSK-Leuk1; Fig. 2A). To confirm that this effect was not unique to this cell system, we also investigated whether TS could induce COX-2 protein in two other cell lines (1483 and H2122) derived from the human aerodigestive tract. TS induced COX-2 in each of these cell lines (Fig. 2B and C). Induction of COX-2 was observed after 6 hours of exposure to TS and persisted for at least 24 hours (Fig. 2D). Notably, TS-mediated induction of COX-2 protein was associated with increased synthesis of PGE<sub>2</sub> (Fig. 2E).

To further elucidate the mechanism responsible for changes in amounts of COX-2 protein, we measured steady-state levels of COX-2 mRNA by Northern blotting (Fig. 3A and B). TS stimulated the



**Figure 5.** Tobacco smoke induces the expression of EGFR ligands in MSK-Leuk1 cells. **A**, cells were treated with vehicle or TS (0.030 puffs/mL) for up to 4 hours. Amounts of amphiregulin (AR) and TGF- $\alpha$  protein in the medium were determined at the indicated time points by enzyme immunoassay. Columns, means (*n* = 6); bars, SD. \*\*, *P* < 0.01 compared with vehicle. **B**, cells were treated with vehicle or TS (0.030 puffs/mL) for up to 16 hours. RT-PCR analysis was done at different points for AR, TGF- $\alpha$ , HB-EGF, and  $\beta$ -actin mRNAs. STD, standard.

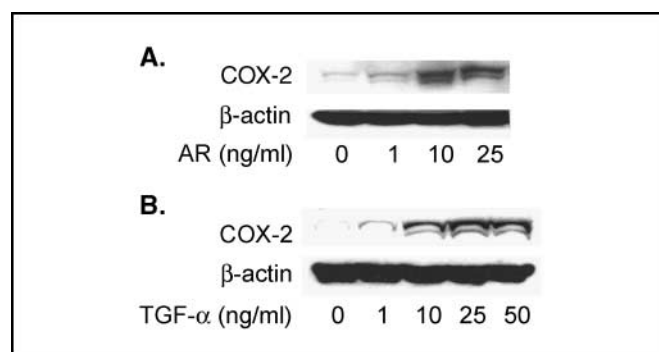


**Figure 6.** Increased levels of amphiregulin and TGF- $\alpha$  are detected in the buccal mucosa of smokers. Biopsies of buccal mucosa were obtained from 9 never smokers and 10 active smokers ( $\geq 10$  pack-years). *A*, RT-PCR was used to determine the amounts of mRNAs for AR and TGF- $\alpha$  in these samples. *B*, results of the data shown in *A* expressed in arbitrary units (a.u.). Columns, means; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with never smokers.

formation of COX-2 mRNA. Differences in levels of COX-2 mRNA could reflect altered rates of transcription. Transient transfections were carried out to determine the effects of TS on COX-2 promoter activity (Fig. 3C). Treatment with TS led to approximately a tripling of COX-2 promoter activity.

Activation of several different signaling cascades including the EGFR→Ras pathway leads to increased COX-2 expression (42). Given this background, we investigated whether TS-mediated induction of COX-2 was EGFR dependent. Accordingly, the effects of TS on levels of phospho-EGFR were measured. TS stimulated EGFR tyrosine kinase activity (Fig. 4A). Subsequently, experiments were carried out to determine whether the activation of EGFR was causally linked to TS-mediated induction of COX-2. Importantly, an inhibitor of EGFR tyrosine kinase activity (AG1478) blocked TS-mediated induction of COX-2 (Fig. 4B). This implies that the induction of COX-2 by TS is dependent on EGFR signaling. Next it was important to begin to define the mechanism by which TS activated EGFR. Activation of EGFR can be growth factor dependent in the absence of exogenous growth factor. Hence, we investigated whether ligand binding was involved in TS-mediated stimulation of EGFR tyrosine kinase activity. We found that antibody blockade of the ligand-binding site abrogated TS-mediated induction of COX-2 (Fig. 4C). This finding can potentially be explained if TS treatment stimulated the release of EGFR ligands. In fact, treatment with TS led to increased amounts of both amphiregulin and TGF- $\alpha$  protein in the medium (Fig. 5A). This effect occurred rapidly, within 1 hour of TS exposure. RT-PCR was

carried out to determine whether the observed increase in amounts of amphiregulin and TGF- $\alpha$  protein in the medium could reflect at least, in part, enhanced gene expression. Exposure to TS led to a marked increase in amounts of amphiregulin and TGF- $\alpha$  mRNAs (Fig. 5B). However, TS-mediated induction of amphiregulin mRNA (Fig. 5B) was first observed only after 2 hours of treatment, whereas



**Figure 7.** Treatment with either amphiregulin or TGF- $\alpha$  induced COX-2. *A*, lysate protein was from MSK-Leuk1 cells treated with vehicle or the indicated concentration of AR (1-25 ng/mL) for 6 hours. *B*, lysate protein was from MSK-Leuk1 cells treated with vehicle or the indicated concentration of TGF- $\alpha$  (1-50 ng/mL) for 6 hours. In *A* and *B*, cellular lysate protein (100  $\mu$ g/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed sequentially for COX-2 and  $\beta$ -actin.

increased release of amphiregulin protein (Fig. 5A) occurred within 1 hour of TS exposure. This means that altered gene expression of amphiregulin cannot explain the rapid release of this ligand into the medium. In contrast to its effect on amphiregulin and TGF- $\alpha$ , TS did not affect levels of HB-EGF mRNA. In an effort to translate these *in vitro* findings to the clinical setting, we evaluated levels of amphiregulin and TGF- $\alpha$  mRNAs in the oral mucosa of active smokers versus never smokers. The amounts of both amphiregulin and TGF- $\alpha$  in oral mucosa were significantly greater in active smokers than in never smokers (Fig. 6). Finally, experiments were carried out to confirm that amphiregulin and TGF- $\alpha$  themselves induce COX-2. As shown in Fig. 7, treatment of MSK-Leuk1 cells with either exogenous amphiregulin or TGF- $\alpha$  resulted in dose-dependent induction of COX-2.

## Discussion

In this study, we have shown that levels of COX-2 are increased in the oral mucosa of active smokers versus never smokers. Consistent with this finding and previous reports (43, 44), treatment with TS induced COX-2 in cell lines derived from the human aerodigestive tract. Several observations support a critical role for EGFR in TS-mediated induction of COX-2. Treatment with TS stimulated the phosphorylation of EGFR. Two different types of evidence have firmly established a causal link between TS-mediated activation of EGFR and the induction of COX-2. Treatment with AG1478, an inhibitor of EGFR tyrosine kinase activity, blocked the induction of COX-2 by TS. Moreover, antibody blockade of the ligand-binding site of EGFR abrogated TS-mediated induction of COX-2. To our knowledge, this study provides the first evidence that TS-mediated induction of COX-2 is EGFR dependent. Notably, these findings are consistent with previous evidence that activation of EGFR signaling can drive COX-2 transcription and PG biosynthesis (5, 45, 46).

Recently, tobacco smoke was found to stimulate the phosphorylation of EGFR in lung epithelial cells (47). This was a consequence of smoke-induced activation of metalloproteinase activity that, in turn, led to cleavage of transmembrane amphiregulin with shedding of active ligand (47). Subsequently, the released mature ligand stimulated EGFR tyrosine kinase activity by an autocrine or paracrine mechanism. Our finding that brief treatment with TS stimulated the release of amphiregulin protein before any change in amounts of amphiregulin mRNA was observed is consistent with this mechanism. Stimulation of G-protein-coupled receptors has been reported to cause metalloproteinase-mediated cleavage of proamphiregulin resulting in transactivation of EGFR (48). Hence, it will be of interest to determine whether G-protein-coupled receptors are involved in TS-mediated induction of COX-2.

In this study, we also showed that exposure of oral epithelial cells to tobacco smoke markedly induced mRNAs for amphiregulin and TGF- $\alpha$ . To our knowledge, this effect of tobacco smoke has not been previously reported. Consistent with these *in vitro* findings, increased amounts of amphiregulin and TGF- $\alpha$  mRNAs were also found in the oral mucosa of active smokers versus never smokers. This finding strongly suggests that the inductive effects of tobacco smoke observed *in vitro* translate to humans *in vivo*. Taking these data together, we postulate that tobacco smoke can stimulate both the synthesis and release of ligands of the EGFR. Given the importance of EGFR in driving mitogenesis, these results provide mechanistic insights that may help to explain the increase in cell proliferation observed in the aerodigestive tracts of active smokers (49, 50).

Cross-talk exists between EGFR and COX-2. As detailed above, activation of EGFR signaling leads to increased amounts of COX-2 and enhanced production of PGE<sub>2</sub> (5, 45, 46). Importantly, there is also growing evidence that COX-2-derived PGs can activate EGFR signaling (51, 52). Therefore, exposure to tobacco smoke may initiate a positive feedback loop whereby activation of EGFR results in enhanced expression of COX-2 and increased synthesis of PGs. This leads, in turn, to a further enhancement of EGFR activity. These effects could also enhance the mutagenicity of tobacco smoke. For example, COX-2 can convert a broad array of carcinogens including polycyclic aromatic hydrocarbons in tobacco smoke to reactive metabolites, which form mutagenic DNA adducts (31, 32). It is possible, therefore, that tobacco smoke-mediated induction of COX-2 will amplify the effect of a given dose of tobacco smoke on tumor initiation. Moreover, conversion of DNA adducts to mutations can only occur in proliferating cells (53, 54) and activation of EGFR signaling or stimulation of PG biosynthesis enhances cell proliferation (24, 42, 55), which, in turn, should increase the mutagenicity of tobacco smoke. Selective inhibitors of COX-2 and EGFR are clinically available. This study provides a mechanism-based rationale for evaluating whether a selective inhibitor of COX-2 or EGFR tyrosine kinase used alone or in combination can prevent or delay the onset of tobacco-related malignancies of the aerodigestive tract.

## Acknowledgments

Received 9/1/2004; revised 10/25/2004; accepted 11/10/2004.

**Grant support:** NIH grants 1 R01 CA82578, P01 CA106451, T32 CA09685, and N01-CN-35107, Pfizer Inc., the Cancer Research and Prevention Foundation, and the Center for Cancer Prevention Research.

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We thank Dr. Assieh Melikian for assistance in preparing saline extracts of tobacco smoke.

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