Celecoxib reduces pulmonary inflammation but not lung tumorigenesis in mice

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Cyclooxygenase (COX) enzyme expression is elevated in human and rodent lung tumors, and non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin reduce lung tumor formation in mice. These observations, along with the well-characterized protection that NSAID treatment engenders for colon cancer, have prompted clinical trials testing whether celecoxib, a COX-2-specific inhibitor, can prevent lung cancer development in populations at high risk. Protection by celecoxib in murine models of pulmonary inflammation and lung tumorigenesis has not yet been evaluated, however, and we now report such studies. Chronic administration of butylated hydroxytoluene (BHT) to mice stimulates pulmonary inflammation characterized by vascular leakage and macrophage infiltration into the air spaces, increased PGE2 production, and translocation of 5-lipoxygenase (5-LO) from the cytosol to the particulate fraction. Dietary celecoxib limited macrophage infiltration, abrogated PGE2 production and reduced particulate 5-LO content. Celecoxib and aspirin were ineffective at preventing lung tumorigenesis in a two-stage carcinogenesis protocol in which 3-methylcholanthrene administration is followed by chronic BHT. Celecoxib also did not reduce the multiplicity of lung tumors after induction by urethane; lung tumors in celecoxib-treated mice were larger than those in mice that did not receive celecoxib. Tumors induced in celecoxib-fed mice contained 60% less PGE2 than tumors in mice fed control diets, so reducing lung PGE2 levels was insufficient to prevent lung tumor formation. As the production of eicosanoids in addition to PGE2 is also inhibited by celecoxib, and as celecoxib has COX-independent interactions, its effects on tumor formation may vary in different organ systems.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the US (1). Prevalence of non-small cell lung carcinoma, including its most common subtype, adenocarcinoma (AC), is increasing in both smokers and non-smokers (2). AC has usually metastasized before clinical symptoms become apparent, thereby reducing treatment options (3). Consequently, the most effective means to combat AC may be chemopreventive strategies (4). Optimism for such strategies developed after recent demonstrations that NSAIDs prevent colon cancer. COX enzymes are rate-limiting for the production of pro- and anti-inflammatory prostaglandin mediators. NSAIDs that inhibit both COX-1 and COX-2, as well as COX-2-specific inhibitors such as celecoxib, induce colorectal adenoma regression in humans (5) and prevent the formation of colorectal adenomas and cancers in animals (6,7). Successful application of these drugs to colon cancer has prompted their evaluation for other malignant diseases (8,9).

Mice develop lung tumors with similar histogenic and molecular features to human AC, providing a model for elucidating events that influence carcinoma development (10–12). Considerable evidence supports a role of inflammation in mouse (13–16) and human (17,18) AC progression. COX-1 and COX-2 enzyme expression is elevated in chemically induced pulmonary tumors (14,19–21), suggesting that COX metabolites formed during dysregulated lung inflammation enhance tumor formation. The COX pathway thus represents a reasonable target for therapeutic intervention. Consistent with this notion, aspirin and the COX-2-specific inhibitor, NS398, inhibited mouse lung tumorigenesis induced by chronic administration of the tobacco carcinogen, NNK (20,22,23).

Carcinogenesis elicited by single agents may not model the onset of human AC as closely as multiple inciting agents. In a two-stage carcinogenesis protocol, BHT is repetitively administered to mice following a single injection of the cigarette carcinogen, MCA (24). The hypothesized mechanism is that MCA induces mutations in K-ras (25) and BHT treatment promotes the selective clonal expansion of these initiated cells (16). Distinct initiation and promotion phases recapitulate the sequence of events leading to smoking-induced human lung cancer, consisting of field cancerization followed by the actions of promoting agents (26). BHT is metabolized in mouse lungs to harmful highly reactive and oxidative species (27) that cause alveolar type 1 cell death followed by compensatory hyperplasia of type 2 cells to replace dead type 1 cells (28). With continued BHT treatment, mice develop a chronic inflammatory response in the absence of further lung injury, comprised of edema, macrophage recruitment into the alveoli and sustained elevation of both COX enzymes in the bronchiolar and alveolar epithelia and in alveolar macrophages (13–16).

Non-carcinogenic pneumotoxicants, such as BHT, that induce chronic inflammation provide a means to evaluate how pulmonary inflammation encourages neoplastic development. We recently demonstrated that aspirin reduces BHT-induced chronic lung inflammation in mice (16). As COX-2 is particularly associated with injury and inflammation, we examined whether the effect of aspirin was primarily due to inhibition
of COX-2 by determining whether celecoxib was similarly effective. Because chronic inflammatory conditions increase cancer risk and COX-2 has been implicated in tumor promotion in other organs (29,30), we evaluated the efficacies of celecoxib and of aspirin for limiting two-stage carcinogenesis. To compare these results with single agent carcinogenesis studies, we determined whether celecoxib prevented tumorigenesis induced by a single urethane injection. Celecoxib inhibited BHT-induced chronic lung inflammation, but the hypothesis that the anti-inflammatory activities of celecoxib and aspirin would reduce mouse lung tumorigenesis was not supported.

Materials and methods

Animals
Male BALB/cBy and A/J mice, 5–6 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the University of Colorado Center for Laboratory Animal Care. Mice were fed AIN-76A chow (Dyets, Bethlehem PA), and in some experiments this was supplemented with either aspirin (ASA; 400 mg/kg of chow) or celecoxib (500, 1000 or 1500 mg/kg of chow). The aspirin dose was based on the study of Bauer et al. (15). These celecoxib doses reduce colon poly and adenoma formation without causing toxicity in mouse models of colon carcinogenesis (31). Mice received either aspirin-supplemented chow for 2 weeks prior to the carcinogenesis regimen or celecoxib-supplemented chow 3 weeks prior to the inflammation and carcinogenesis protocols; pulmonary PGE2 levels in control mice are almost completely abrogated after 3 weeks of dietary celecoxib (Table I). The mice were fed food and water ad libitum and maintained on hardwood bedding under a 12 h light/dark cycle. Food was changed twice weekly, and daily food consumption was monitored. Animals were weighed weekly for the duration of the experiments.

Pharmaceuticals and diets
Celecoxib, provided through an agreement with Pharmacia, and aspirin (Sigma Chemical Co., St Louis, MO) were shipped to Dyets (Bethlehem, PA) and mixed with AIN-76A chow, a purified formulation used in other tumorogenesis/chemoprevention models (32,33). Because it is anti-oxidant free, AIN-76A does not interfere with the metabolism of the inflammatory and carcinogenic agents utilized in our experiments. Fresh chow was shipped monthly, stored at 4 °C, and mice were fed these chows for the duration of the experiments. BHT (Sigma) and MCA (Sigma) were dissolved in corn oil (Mazola) prior to injection.

Plasma celecoxib levels
The assay protocol of Paulson et al. (34) was modified for celecoxib extraction and HPLC analysis. Plasma was added to 1 M phosphoric acid, and solid phase extraction conducted using 50 mg Isolute HCX mixed mode non-polar/cation exchange columns (Jones Chromatography, Lakewood, CO) pre-treated with acetonitrile and water. Following sample addition, the columns were washed with water and allowed to dry. Celecoxib was eluted with 0.6% ammonium hydroxide in methanol, dried, re-suspended in 50% acetonitrile and 100 μl injected into the HPLC system. Final plasma concentrations were calculated based on comparison with a standard curve that was linear over a range of 10–2500 nM. These assays were performed by the University of Colorado Pharmacokinetics Core Facility.

BHT induction of chronic lung inflammation
Prior to performing the drug studies, we compared commercial and AIN-76A chows to determine if the absence of anti-oxidants in AIN-76A influenced sensitivity to BHT. A hallmark of BHT treatment of mice is a well-defined pattern of weight loss that is maximal 6 days following a single BHT injection (35). BALB mice fed AIN-76A chow were more sensitive to BHT than mice maintained on standard chow, as indicated by weight loss and increased mortality (data not shown). Enhanced sensitivity to carcinogens when mice are fed AIN-76A chow compared with standard laboratory chow is consistent with data reported by others (36). To induce pulmonary inflammation with minimal mortality, the mice received an initial BHT dose of 125 mg/kg body weight i.p. Subsequent BHT doses, administered once per week for an additional 3 weeks, were increased in 25 mg/kg increments (i.e. 150, 175 and 200 mg/kg).

Bronchoalveolar lavage (BAL) analysis
Six days following the final BHT injection, mice were anesthetized, the trachea cannulated and the lungs lavaged with 3×1 ml of phosphate-buffered saline containing 0.6 mM EDTA, as described previously (16). The protein content of the initial milliliter of lavage fluid was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA); BAL protein content was used to estimate vascular permeability. Inflammatory cell infiltration was determined by pooling the lavaged samples and counting cells using a hemocytometer. Differential cell counts based on cell morphology as determined following a modified Wright’s stain (performed by the University Hospital Clinical Laboratory, Denver, CO) were used to identify the infiltrating cells as macrophages, lymphocytes, neutrophils or eosinophils. Alveolar macrophage content in BAL fluid is maximal 6 days after BHT, and 95% of the cells present in the BAL from control and BHT-treated mice are macrophages (16).

Western blotting of 5-lipoxygenase (5-LO)
Preparation of lung extracts and subsequent western blotting was performed as described previously (14). Briefly, cytosolic and particulate protein fractions were blotted and incubated with a mouse monoclonal antibody to 5-LO (1:500; BD Transduction Laboratories, Lexington, KY), and then incubated with a mouse IgG secondary antibody (1:10 000, Santa Cruz Biotechnology, Santa Cruz, CA). Even protein loading of the gels was confirmed by staining the membranes with 0.1% Ponceau S (Fisher Biotech, Fair Lawn, NJ) in 5% acetic acid.

ELISA immunoassays for pulmonary PGE2
Lungs were perfused through the pulmonary artery with 0.9% saline and removed. The large right lobe was weighed and homogenized in 3 vol/wt of 100% methanol. Individual samples were stored at −80 °C until ELISA immunoassays (Cayman Chemical, Ann Arbor, MI) were performed in duplicate at a 1:25 sample dilution exactly according to manufacturer’s instructions. To analyze PGE2 in isolated lung tumours, dissected tumours were pooled from a single mouse, homogenized in 200 ml of 100% methanol and assayed as above at a 1:50 sample dilution. As the tumors were of similar size (Figure 5B), calculations of PGE2/tumor were determined by dividing total PGE2 by the number of tumors in the sample.

Two-stage carcinogenesis
Celecoxib
BALB mice were injected with 15 mg/kg MCA and fed AIN-76A or AIN-76A + celecoxib (500 mg/kg in chow) for 3 weeks; thereafter, mice were administered once per week doses of BHT for 6 weeks. The first dose was 125 mg/kg; the second BHT dose was raised to 150 mg/kg, but significant mortality was observed. Consequently, the third through to the sixth injection remained at 125 mg/kg. Tumors were enumerated and sized using a digital caliper (Mitutoyo, Tokyo, Japan) 20 weeks following the MCA injection.

Aspirin
BALB mice were fed AIN-76A or AIN-76A + 400 mg/kg aspirin chow for 2 weeks prior to MCA (15 mg/kg) administration. One week later, mice received BHT on a weekly basis for 6 weeks. The first dose was 150 mg/kg, and subsequent doses were 200 mg/kg; no morbidity in mice on an aspirin-containing diet was observed with these BHT doses. Lung tumors were enumerated and sized 20 weeks following the MCA injection.

Urethane carcinogenesis
A/J mice were injected i.p. with 1 g/kg of urethane (Sigma), and 2 days later were fed AIN-76A chow alone or supplemented with 500 or 1000 mg/kg celecoxib. Tumors were enumerated and sized 16 weeks following urethane
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Fig. 1. Plasma levels of celecoxib following dietary administration. Celecoxib (500, 1000 or 1500 mg/kg in AIN-76A chow) was administered to control (closed squares) or BHT-treated (closed triangles) BALB mice. Most animals treated with 1500 mg/kg celecoxib died. After 3 weeks on dietary celecoxib, blood samples were obtained and celecoxib plasma levels determined by HPLC. Data represent mean ± SEM from five to 10 animals per group.

Fig. 2. BHT and celecoxib modulation of pulmonary 5-LO expression in BALB mice. (A) Immunoblot and (B) densitometry of cytosolic 5-LO; (C) immunoblot and (D) densitometry of particulate 5-LO. Each band on the western blots represents pulmonary 5-LO content from a lung extract from one mouse. Data represent mean ± SEM from three mice per group.

Results

Effects of celecoxib on BHT-induced chronic pulmonary inflammation

Celecoxib plasma levels increased linearly as dosing was increased to 1000 mg/kg (Figure 1). BALB mice that were fed 1500 mg/kg celecoxib lost weight and many died, especially in the longer tumorigenesis studies (data not shown). Necropsy on one moribund mouse (performed by the University of Colorado Center for Laboratory Animal Care) revealed renal tubular degeneration as the most likely cause of death. Administration of four weekly doses of BHT did not affect celecoxib plasma levels (Figure 1).

Table I summarizes the effects of celecoxib on BHT-induced lung inflammation; extensive histological description of BHT-induced inflammation has been published (15,16). Chronic BHT treatment elicited an inflammatory response in which the number of BAL macrophages rose nearly 5-fold over vehicle-treated mice; 1000 mg/kg celecoxib attenuated this response by 41%. Vascular permeability as assessed by measurements of BAL protein content increased nearly 5-fold by BHT; celecoxib did not significantly perturb this increase. BHT treatment of mice induced nearly 2-fold higher PGE2 concentrations in lung extracts; both doses of celecoxib abrogated this BHT-induced increase.

Similar BHT-induced elevations in inflammatory cell influx and BAL protein have been described previously (15,16). Because COX inhibition should theoretically divert arachidonate metabolism to alternative pathways, we also examined 5-LO, an important enzyme in leukotriene synthesis. BHT decreased cytosolic 5-LO protein expression and increased membrane-associated 5-LO (Figure 2). Celecoxib did not affect the amount of cytosolic 5-LO detected by western blotting, but diminished the BHT-induced elevation of membrane-associated 5-LO, suggesting that celecoxib could influence leukotriene production.

Effects of celecoxib and aspirin on lung tumorigenesis

Although celecoxib (Table I, Figure 2) and aspirin (16) attenuate pulmonary inflammation, neither drug affected two-stage carcinogenesis. Administration of MCA by itself induced few tumors; most mice were tumor-free, while a few had one or two tumors, consistent with previous results (15,16,24). However, when multiple BHT dosing followed the MCA injection, all mice developed lung tumors (Figures 3A and 4A). Tumor multiplicities averaged 7 tumors/mouse in the celecoxib experiments and 20 tumors/mouse in the aspirin studies. These multiplicities were unaffected by treatment with 500 mg/kg celecoxib or 400 mg/kg aspirin (Figures 3A and 4A). Many of the mice receiving 1500 mg/kg celecoxib died (12 out of 20); there was no effect of celecoxib on the animals that lived. Unexpectedly, aspirin and celecoxib increased tumor size by 17 and 40%, respectively (Figures 3B and 4B).

Long-term administration of 1000 mg/kg celecoxib was toxic
Effects of dietary celecoxib on MCA/BHT lung carcinogenesis in BALB mice. (A) Tumor multiplicity. Data represent tumor numbers from individual mice; mean number of tumors/mouse is shown by horizonal bars. Mice per group: MCA/H11001BHT 14; MCA/H11001BHT/1200 mg/kg celecoxib/H11001 12. (B) Tumor size. Each data point is the size of one tumor. Horizontal bars represent mean tumor size. **P < 0.05 v MCA/H11001BHT, no celecoxib.

to urethane-treated A/J mice. At 16 weeks following urethane injection, only four out of 20 mice in the 1000 mg/kg celecoxib group survived, while 14 out of 20 of the 500 mg/kg celecoxib group and 20 out of 20 of the control (AIN-76A) group survived. Therefore, comparisons were made using the mice from the AIN-76A and 500 mg/kg celecoxib-treated groups only. Celecoxib did not affect urethane-induced tumor number (Figure 5A) or size (Figure 5B). To verify that celecoxib inhibited tumor-associated COX-2 activity, PGE2 concentrations were assayed in tumors collected from urethane-treated mice fed AIN-76A alone or supplemented with celecoxib (Figure 6). The tumors in celecoxib-treated mice had 60% less PGE2 than tumors from control mice.

Discussion
We observed pharmacogenetic differences in the metabolism and toxicity of celecoxib. Analogously, plasma concentrations of COX inhibitors after oral administration of a standard dose are variable in humans (37). Although C57BL/6J (B6) mice have been reported to safely consume celecoxib doses as high as 3200 mg/kg (32,34), BALB mice tolerated up to 1000 mg/kg (Figure 1). A dose of 1500 mg/kg celecoxib caused weight loss and mortality. The cause of death in one animal was renal failure, a complication reported previously to result from celecoxib administration to humans (38) and in COX-2 null mutant mice (39). A 1500 mg/kg celecoxib dose caused mortality in hairless SKH-1 mice, although this was dependent on UV exposure (40). These strain differences in celecoxib pharmacokinetics and toxicity emphasize the importance of pharmacogenetic considerations when testing effects of celecoxib.

Celecoxib inhibited several inflammatory parameters induced by chronic BHT treatment of mice, including macrophage recruitment and elevated pulmonary PGE2 levels (Table I); aspirin also inhibited these inflammatory parameters (16). Western blotting of lung tissue also revealed that cytosolic 5-LO decreased and membrane-associated 5-LO increased upon BHT treatment (Figure 2). Activation of alveolar macrophages stimulates 5-LO translocation from the cytosol to the perinuclear membrane in these cells (41). Our data are consistent with these published results as well as with data localizing 5-LO to the nucleus of rat alveolar macrophages.
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Fig. 5. Effects of celecoxib on urethane lung carcinogenesis in A/J mice. (A) Tumor number. Data represent number of tumors per mouse; horizontal bars, mean number of tumors per mouse. (B) Tumor size. Bars represent means; error bars, SEM. Mice per group: no celecoxib/H11005 19; 500 mg/kg celecoxib/H11005 12.

(42). Celecoxib abrogated the increased particulate 5-LO content without changing cytosolic levels. The reduced macrophage recruitment into the alveoli caused by celecoxib would be predicted to decrease membrane-associated 5-LO in whole lung extracts. This hypothesis is mitigated, however, by the observation that BHT-induced elevation in particulate 5-LO was abrogated at a celecoxib dose (500 mg/kg) that did not reduce the number of BAL alveolar macrophages (Table I). Celecoxib might interfere with macrophage activation without influencing macrophage recruitment; this was not assessed. Products of 5-LO catalysis, such as 5-HETEs, have been implicated in cancer development (43).

This is the first report that examines whether celecoxib inhibits early experimental respiratory carcinogenesis and is noteworthy because of the imminence of clinical trials using celecoxib for lung cancer chemoprevention. Although celecoxib and aspirin (16) attenuated pulmonary inflammation by reducing macrophage infiltration, neither drug prevented lung tumorigenesis; however, both drugs increased the size of tumors induced by MCA/BHT. This implies that modulation of macrophage numbers per se is not sufficient to regulate tumor development. The small but significant increases in tumor size induced by both aspirin and celecoxib suggest that macrophages and their products have both beneficial and detrimental pulmonary actions within this carcinogenesis protocol. Increased tumor size could result from altering the balance between the rates of cell proliferation and apoptosis; our experiments were not designed to assess this hypothesis. Celecoxib decreased PGE2 content in urethane-induced lung tumors, so COX-2 activity was clearly inhibited by including the drug in the diet, but this was not sufficient to diminish tumorigenesis. Tumor size was unaffected by celecoxib in urethane-induced carcinogenesis. Our studies address the efficacy of NSAIDs on adenoma formation, and may not be predictive of any effects of celecoxib and aspirin on other forms of lung cancer, such as small cell and squamous cell carcinomas, or on later stages of adenocarcinoma formation and metastasis. Indeed, celecoxib inhibited the pulmonary colonization by injected Lewis lung carcinoma cells, supporting its efficacy at preventing metastasis to the lung (32). Future experiments will examine effects of COX inhibition on later stages of mouse adenocarcinoma growth.

Table II summarizes published attempts to influence mouse lung carcinogenesis with anti-inflammatory compounds. Raising plasma levels of corticosterone, the endogenous murine glucocorticoid, lowered urethane-induced tumor multiplicity; consistent with this, adrenalectomy elevated tumor number (44). The synthetic glucocorticoid, dexamethasone, inhibited lung tumorigenesis induced by various agents (45–49). Aerosolized budesonide, another synthetic glucocorticoid, abrogated BP-induced lung tumor formation (50). Analogous to our results with MCA/BHT (Figure 4), aspirin did not affect lung tumor induction by BP (51), NNK (47) or cigarette smoke (47). However, lung tumor formation was diminished by aspirin upon chronic administration of NNK (20,22,23). The lack of aspirin chemoprevention in some studies versus this positive effect may reflect the carcinogenesis protocols used, i.e. chronic NNK might be more sensitive to modulation by...
Table II. Summary of anti-inflammatory drug effects on mouse lung tumorigenesis

<table>
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<tr>
<th>Drug</th>
<th>Carcinogen</th>
<th>Strain</th>
<th>Effect</th>
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<td>Dexamethasone</td>
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<td></td>
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<td>NNK</td>
<td>A/J</td>
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<td></td>
<td>Cigarette smoke</td>
<td>A/J</td>
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<td>47</td>
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<td>A/J</td>
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<td>FVB</td>
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<td>A/J</td>
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<td>MCA + BHT</td>
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<td></td>
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<td>20,22,23</td>
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<tr>
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<td>NNK (chr)</td>
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<sup>a</sup>L, decreased tumor multiplicity; –, no effect.
<sup>b</sup>ENU was administered transplacentally.
<sup>c</sup>Budesonide was applied by aerosolization.
<sup>d</sup>NNK was administered chronically.

..aspirin than bolus injections of carcinogens. Carcinogenesis protocols in which aspirin was ineffective also employed chronic dosing, however; 5 months of exposure to cigarette smoke (47) and six weekly injections of the BHT promoting agent (Figure 3). The differences are not due to gender as Adriamennsen et al. (51) and Castonguay et al. (20,22,23) both used female mice, nor are strain differences involved as all four studies used A/J mice. With regard to other NSAIDs, indomethacin inhibited lung carcinogenesis induced by either NNK (22) or urethane (21), and sulindac inhibited induction with chronic NNK (22,52) or VC (48). Naproxen had no effect in the chronic NNK model (52). These chemopreventive efficacies of indomethacin and sulindac are consistent with a role of COX-1, in addition to COX-2, in lung tumor development. The only report using a COX-2-specific NSAID for lung tumor chemoprevention in mice, besides our negative findings with celecoxib (Figure 3A), was inhibition by NS398 in the chronic NNK model (23). This difference does not reflect celecoxib inactivity as dietary celecoxib reduced PGE<sub>2</sub> synthesis in tumors (Figure 6). Of interest, given that celecoxib reduces the BHT-induced rise in particulate 5-LO, is that the lipooxygenase inhibitors, NDGA and A79175, lowered lung tumor induction by urethane (21) and chronic NNK (53), respectively.

The COX pathway produces not only pro-inflammatory prostaglandins like PGE<sub>2</sub>, but also prostaglandins such as PGI<sub>2</sub> that are anti-inflammatory in the lungs (54). Indeed, elevating endogenous pulmonary PGI<sub>2</sub> levels in mice with a lung-specific PGIS transgene decreased lung tumorigenesis in both of the carcinogenesis protocols used herein (55). Thus, the cumulative effects of COX inhibitors on lung physiology are not obviously predictable. NSAIDs have biological activities not involving COX inhibition (56), such as serving as ligands that activate the PPAR steroid receptor family (57) or inhibit NF-κB (58). Lung cancer epithelium, as well as the surrounding stroma, exhibits high PPAR expression (59). Our data emphasize the importance of cellular context in chemoprevention. Diminishing COX activity clearly inhibits mouse intestinal cancer development (6), but may not affect mouse lung tumorigenesis. Comparing the relative chemopreventive potencies of the sulfide and sulfone metabolites of sulindac illustrates such organ-specificity. Sulindac sulfide inhibits COX activity and PGE<sub>2</sub> formation, but the sulfone metabolite does not (60). Sulindac was much more effective than the sulfone in preventing colon tumors (61), but much less effective than the sulfone in protecting against mouse lung tumorigenesis (53). Collectively, the data demonstrate that the effects of COX-2 inhibition are complex and emphasize the necessity of preclinical testing in target organs prior to initiating clinical trials. Our studies should be interpreted as cautious when devising chemoprevention strategies for high-risk populations, such as ex-smokers.

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