Decreased lung tumorigenesis in mice genetically deficient in cytosolic phospholipase A2

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Epidemiological investigations suggest that chronic lung inflammation increases lung cancer risk. Pharmacologic and genetic evidence in mouse models indicates that lipid mediators released during pulmonary inflammation enhance lung tumor formation. Cytosolic phospholipase A2 (cPLA2) catalyzes arachidonic acid (AA) release from membrane phospholipids. AA can then lead to the synthesis of several classes of lipid mediators, including prostaglandins (PGs), which are synthesized via cyclooxygenase (COX). In the COX-dependent pathway, AA is oxidatively cyclized to form prostaglandin H2, which is then reduced to prostaglandin E2 (PGE2) or prostaglandin I2 (PGI2). The signaling pathways of these two eicosanoids are distinct, with PGE2 causing vasodilatation and smooth muscle contraction, while PGI2 causes vasodilatation and smooth muscle relaxation (1). In pulmonary tissues and the cellular localization of cPLA2, an increase that was significantly blunted in tumors from cPLA2 null mice. These results demonstrate a role for cPLA2 in mouse lung tumorigenesis that may be mediated by decreased prostaglandin synthesis.

Abbreviations: AA, arachidonic acid; B6, C57BL/6J mice; COX, cyclooxygenase; cPLA2, cytosolic phospholipase A2; IH, immunohistochemistry; 6-keto PGF1α, 6-keto prostaglandin F1α; mPGES, microsomal PGE2 synthase; PAF, platelet-activating factor; PGE2, prostaglandin E2; PGI2, prostaglandin I2.

Materials and methods

Mice
cPLA2 null mice in a C57BL/6J-129/Sv (B6-129/Sv) chimeric background and their wild-type littermates were bred in the animal facilities at Harvard University, Denver VA Medical Center, and the University of Colorado Health Sciences Center. Since offspring produced by homozygous knockouts are non-viable (18), breeding was conducted with heterozygotes. Mice were fed Harlan Teklad 22/5 rodent chow (Harlan, Madison, WI), given water ad libitum, and housed on hardwood bedding with a 12-h light/12-h dark cycle.
in a climate-controlled facility. Carcinogenesis was performed using 10–15-week-old mice of both genders.

**Kras allele determination**

Certain inbred strains of mice have a *Kras* allele that renders them sensitive to chemically induced lung tumorogenesis (e.g. A/J, 129/Sv), while others have a resistant allele and develop very few or no lung tumors (e.g. B6) (19). Mice with one copy of a 37 bp sequence in *Kras* intron 2 are sensitive, while strains with two copies arranged in tandem are resistant (20). Since the cPLA2 knock-out mouse was generated as a B6/129 chimera, the *Kras* genotype was determined; mice bearing the resistant *Kras* allele were removed from experimental analysis so that only the contribution of the presence or absence of functional cPLA2 was assessed. Genomic DNA was isolated and PCR reactions using primers for examining the *Kras* intron 2 polymorphism were performed as described (20).

**Lung carcinogenesis**

Because the B6/129 genetic background of the cPLA2 /-- mice consists of resistant B6 alleles and 129/Sv alleles that convey intermediate sensitivity (21), multiple injections of urethane were used to induce lung tumor formation in cPLA2 /-- and +/- littermates; this carcinogenesis procedure induces several tumors in B6/129 chimeras (22). Mice were injected i.p. once per week for 6 weeks with 1 mg urethane (Sigma, St Louis, MO) dissolved in 0.9% NaCl/g body wt, weighed weekly, and observed daily for the duration of the study to assess their health. Nineteen weeks after the initial carcinogen injection, tumors were enumerated using fresh tissue examined under a dissecting microscope, and tumor diameters measured with digital calipers. The genetic identity of the mice (null or wild-type for cPLA2) was not revealed until after tumor multiplicities and sizes were determined. Mice were also subjected to this carcinogenesis regimen to obtain tissue samples for immunohistochemistry and immunoblotting.

**Immunohistochemistry (IH)**

Lung tissue sections were prepared for IH as described (7). Briefly, the lungs of urethane-treated mice were perfused through the pulmonary artery with saline and fixed by inflation with 10% formalin. Lungs were dehydrated, embedded in paraffin, and cut into 4 μm sections that were rehydrated. Endogenous endoperoxidase activity was inhibited by incubation with 3% H2O2 in methanol for 15 min, followed by antigen retrieval using warm 0.1% citrate buffer, pH 6.0. In these experiments, a mouse monoclonal cPLA2 antibody (Santa Cruz, Santa Cruz, CA) was used at a 1:50 dilution for immunostaining after blocking endogenous mouse immunoglobins using the Mouse-On-Mouse kit (Vector Laboratories, Burlingame, CA); goat polyclonal COX-1 and COX-2 antibodies (Santa Cruz) were used at a 1:2000 dilution for each (7). A goat polyclonal mPGES antibody (Cayman Chemical, Ann Arbor, MI) was used at a 1:500 dilution. Samples were treated with biotin-conjugated anti-mouse IgG or anti-goat IgG secondary antibody (Vector) followed by peroxidase-conjugated streptavidin tertiary antibody complex (Vector). 3,3-Diaminobenzidine (Sigma) was used as the peroxidase substrate for cPLA2, COX-1, COX-2 or mPGES detection and hematoxylin (Sigma) was the counterstain. As a negative control for cPLA2 staining, a tumor-bearing tissue sample from a cPLA2 null mouse was immunostained for cPLA2. As a negative control for mPGES staining, mPGES primary antibody was treated with a blocking peptide and then incubated with slides to demonstrate antibody specificity.

**Immunoblotting**

Lung tissue from untreated age-matched controls and tumor samples from urethane-treated B6/129 mice were homogenized in 20 mM HEPES, pH 7.5 buffer containing 10% glycerol and protease inhibitors (2 mM EDTA, 2 mM EGTA, 5 μg/mL aprotinin and 10 μM leupeptin) (7). Soluble and particulate fractions were separated by centrifugation at 16 000 g for 30 min. Particulate proteins were re-suspended in homogenization buffer with 0.1% NP-40 for immunoblotting and COX-1, COX-2 and mPGES; cPLA2 content was examined in soluble fractions. After protein concentrations were determined by the method of Lowry et al. (23), 50 μg of protein/lane applied to 10% cPLA2, COX-1 and COX-2 or 15% mPGES SDS-PAGE. Separated proteins were transferred onto Immobion-P polyvinylidine fluoride membranes (Millipore, Bedford, MA). Primary antibodies used for immunoblotting were the same as those used for IH. For cPLA2 detection, membranes were blocked for 30 min in 15 mM Tris, pH 7.4 buffer containing 150 mM NaCl, 3% powdered milk and 0.1% Tween-20 (Sigma), incubated with primary antibody for 1 h at a 1:300 dilution in blocking solution, and incubated for 1 h with goat anti-mouse alkaline phosphatase secondary antibody (Bio-Rad, Hercules, CA; 1:3000 dilution). Membranes were incubated with Immun-Star chemiluminescent substrate (Bio-Rad) and exposed to CL-XPosure film (Pierce, Rockford, IL). cPLA2 bands at 110 kDa were quantified using UN-SCAN-IT gel digitizing software (Silk Scientific, Orem, UT). COX-1 and COX-2 protein immunoblotting was performed as described previously (7). Membranes were incubated with COX-1 or COX-2 primary antibody (1:800 dilution for each) or mPGES primary antibody (1:500 dilution), washed and incubated with horseradish peroxidase labeled donkey anti-goat secondary antibody (1:20 000 dilution). After treatment with ECL-reagent (Pierce), membranes were exposed to X-ray film and the resulting 72 (COX) or 14 kDa bands (mPGES) quantified as above. To confirm even protein loading of the gels, the membranes were stained with 0.1% Ponceau S (Fisher Biotech, Pittsburgh, PA) in 5% acetic acid.

**PGE₂ and PGI₂ estimation by enzyme immunoassay (EIA)**

PGL₂ is unstable (t1/2 = 3 min) and hydrolyzed non-enzymatically to the more stable 6-keto prostaglandin F₁α (6-keto PGF₁α) metabolite (12). PGE₂ and 6-keto PGF₁α EIA assays were performed according to manufacturer instructions (Cayman Chemical, Ann Arbor, MI). Mice were killed by lethal phenobarbital i.p. injection 19 weeks after carcinogen treatment, the lungs excised, and tumor and uninvolved lung tissue separated by dissection. Tumors dissected from a single mouse were pooled and snap frozen. Lung tissue from age-matched, untreated mice (control) were also collected. Samples were homogenized in Earle’s salt solution and an aliquot was removed for protein concentration measurement (Bio-Rad). Lipids were extracted from the remaining samples in 4 vol (w/v) methanol. Samples were centrifuged at 16 000 g for 10 min to remove tissue debris, and the supernatants used

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**Fig. 1.** cPLA₂ as the starting point for generating numerous lipid mediators.
circumstances, the percentage of mice that had any tumors, was 100% for from two independent experiments. Tumor incidence, i.e. allele were removed from the study. Absence of cPLA2 multiplicity in cPLA2 tumor multiplicities were calculated by combining results identified using the Student’s unpaired test. One-way analysis of variance (ANOVA) was used to compare more than two groups and post-hoc Newman-Keuls tests were used to identify differences between groups. Under all circumstances, \( P < 0.05 \) was considered to be significant.

Results

Decreased lung tumorigenesis in cPLA2 null mice

Nineteen weeks following the first of several weekly urethane injections, wild-type littermates developed 16 ± 2 tumors while cPLA2 null mice developed 9 ± 2 tumors/mouse (Figure 2), a 43% reduction (\( P < 0.05 \)). These average tumor multiplicities were calculated by combining results from two independent experiments. Tumor incidence, i.e. the percentage of mice that had any tumors, was 100% for both groups of animals. Two mice homozygous for the resistant Kras allele were removed from the study. Absence of cPLA2 did not affect morbidity or mortality of mice undergoing the carcinogenesis protocol; the good health of cPLA2 null mutants not treated with carcinogens has been observed previously (16). Since null mice did not lose body weight or have their rate of weight gain compromised, the reduced tumor multiplicity in cPLA2 \(-/-\) mice was not due to caloric restriction, a condition that inhibits lung tumor formation (24). Tumor sizes were similar between groups (data not shown).

Immunohistochemical analysis of cPLA2, COX-1, COX-2 and mPGES

To determine whether tumorigenesis or the absence of cPLA2 affected immunolocalization of relevant enzymes, lungs from normal and urethane-induced, tumor-bearing B6/129 mice were examined. In lungs from untreated animals, cPLA2 was localized to bronchiolar and alveolar epithelial cells and to alveolar macrophages; staining was mainly cytoplasmic (Figure 3A). Adenomas arise from bronchiolar non-ciliated Clara cells and from alveolar Type II cells (2). In tumor-bearing animals, cPLA2 was present in epithelial cells adjacent to the tumors and throughout the tumor parenchyma. Parenchymal staining was faint while staining was more intense in tumor-associated macrophages (Figure 3B). The inset in Figure 3(B) demonstrates the lack of cPLA2 immunostaining in a tumor from a cPLA2 null mouse. Similar to cPLA2, mPGES was found in bronchiolar and alveolar epithelial cells and alveolar macrophages in normal lungs (Figure 3C); mPGES was also expressed in smooth muscle cells (data not shown). In tumor-bearing mice, mPGES localized to epithelial cells in adjacent normal tissue, within the tumor parenchyma, and in tumor-associated macrophages (Figure 3D). Pre-incubation of the primary antibody with a blocking peptide demonstrated antibody specificity (inset, Figure 3D). As has been observed previously in A/J (8), BALB (7) and other B6/129 chimeric mice (22), both COX-1 (Figure 3E) and COX-2 (Figure 3F) intensely immunostained within the tumor epithelia, as well as in surrounding uninvolved tissue and macrophages.

Immunoblotting of cPLA2, COX-1, COX-2 and mPGES in normal and neoplastic lung tissue

cPLA2, COX-1 and 2, and mPGES expression was evaluated by immunoblot to quantify enzyme content in extracts from control lung and tumors from urethane-treated B6/129 mice. As expected, bands corresponding to cPLA2 are absent from samples obtained from cPLA2 null mice (Figure 4A). In wild-type littermates, cPLA2 expression increased 2-fold in tumors compared with untreated lungs (Figure 4B). COX-1 and COX-2 expression in tumors increased at least 5-fold over untreated lungs in both cPLA2 null mice and wild-type littermates (Figure 4C and D). Increased COX content was observed previously in tumors that were induced in other inbred strains by carcinogenesis protocols different from that used herein (7,8,25). cPLA2 deficiency does not apparently affect this up-regulation of COX expression. mPGES expression in tumors increased 5-fold in cPLA2 null mice; expression also increased in tumors induced in wild-type littermates, although this was not statistically significant (Figure 4E). Dissected tumors contain both neoplastic epithelial cells and tumor stroma, including macrophages, and the altered enzyme concentrations in these tumor extracts reflects the contribution of all these cells.

PGE2 and 6-keto PGF1α concentrations in lung tissue and tumors from cPLA2 null mice

To determine if decreased prostaglandin production due to diminished AA availability may be at least partly responsible for the decreased number of tumors induced in cPLA2 null mice, prostaglandin levels were measured in lungs from age-matched untreated mice (control) and tumors isolated from both wild-type and cPLA2 null mice (Figure 5). Tumors induced in wild-type mice contained more of both PGE2 and 6-keto PGF1α than control lung tissue, as normalized on a per milligram protein basis. In lung tumors isolated from cPLA2 null mice, these increases were markedly blunted. No significant differences in prostaglandin levels were observed between lungs from untreated wild-type or cPLA2 null mice.

Discussion

Individuals with chronic lung inflammatory diseases such as asthma and chronic bronchitis have increased lung cancer risk (26,27). Anti-inflammatory drugs, such as non-steroidal
Fig. 3. cPLA₂, COX-1, COX-2 and mPGES IH in lungs from untreated and urethane-treated B6/129 mice. (A) cPLA₂ and (C) mPGES immunostained in bronchiolar (B) and alveolar (A) epithelial cells as well as alveolar macrophages (M) in untreated mice. (B) cPLA₂ faintly stained in tumor parenchyma (T), with more pronounced staining in surrounding macrophages (M). Tumors from cPLA₂ null mice show no cPLA₂ staining (inset, B). (D) mPGES was present in tumor parenchyma (T) and tumor-associated macrophages (M). A blocking peptide showed specificity of the mPGES antibody (inset, D). (E) COX-1 and (F) COX-2 immunostained the tumor epithelium (T) and surrounding macrophages (M). A total of five tumors were examined from three mice with similar results. Black bar in (A) represents 50 μm.
anti-inflammatory drug (NSAIDs) that inhibit COX-1 and COX-2 activities (4,8) and lipoxygenase inhibitors that inhibit leukotriene formation (5,6), reduce the multiplicity of lung tumors in mice induced with chemical carcinogens. Transgenic mice that specifically over-express PGIS targeted to the cell types from which tumors arise develop fewer lung tumors in response to carcinogens than wild-type mice (9). A clinical trial that utilizes an inhibitor of COX-2, in addition to other chemotherapies, has shown promising preliminary results in treating early-stage lung cancer (28). The eicosanoid pathway produces potent inflammatory mediators, but pharmacological inhibitors of cPLA2 applicable for clinical studies have not been developed. Genetic ablation of cPLA2 inhibits mouse lung tumorigenesis (Figure 2), providing the first evidence that decreasing cPLA2 function protects against lung cancer.

Cultured cells derived from cPLA2-deficient animals produce very low levels of PGs compared with wild-type cells. Peritoneal macrophages isolated from cPLA2 null mice that were stimulated ex vivo with calcium ionophore or LPS produced markedly less PGE2 than cells isolated from wild-type mice (16,17,29). Steady-state tissue levels of prostaglandins, however, are not altered in these animals (Figure 5). The intracellular concentrations of COX-1, COX-2 and mPGES were also unchanged in the knockout mouse (Figure 4). These data suggest that cPLA2 is not activated in these tissues in the absence of external stimuli; prostaglandins are apparently produced basally by other forms of PLA2, such as secretory sPLA2 or calcium-independent iPLA2. In particular, iPLA2 has been implicated in phospholipid remodeling (30) and is likely to be constitutively active in those tissues in

![Fig. 4](https://academic.oup.com/carcin/article-abstract/25/8/1517/2475936/5)

**Fig. 4.** cPLA2, COX-1, COX-2 and mPGES increase in tumors. Lung tissue and tumors were harvested from age-matched controls and tumor-bearing urethane-treated mice. Samples were prepared from each of three control mice and three tumor-bearing mice. (A) cPLA2, COX-1, COX-2 and mPGES in control (CON) and tumor (TUM) from cPLA2+/+ and −/− mice. (B-E) Densitometric quantitation of protein levels from immunoblots. (*P < 0.05 versus control.)
which it is expressed. Free AA could also be provided through the sequential actions of phospholipase C and diglycerol lipase (31,32).

PGE$_2$ and 6-keto PGF$_{1\alpha}$ levels are up-regulated during tumor formation (Figure 5). Similar findings have been reported in human lung tumors (33), underscoring the similarities between the mouse model and human lung cancer. These increases result from increased activities of cPLA$_2$, COX-1, COX-2 and/or mPGES, which are a consequence, at least in part, of increased expression of these enzymes. Higher levels of expression may be mediated by activation of gene transcription, or could be a consequence of mouse tumors being derived from cells (e.g. Type II), which express higher levels of these proteins than other cell types in normal lung tissue (Figure 4). We propose that increased cPLA$_2$ activity is due to both increased expression in tumors, which we have observed, as well as activation of the enzyme via post-translational pathways. cPLA$_2$ activity can also be regulated by increases in intracellular Ca$^{2+}$ and by protein phosphorylation (34,35). Consistent with a role of prostaglandins in tumorigenesis, less PGE$_2$ and 6-keto PGF$_{1\alpha}$ was detected in tumors from cPLA$_2$ null mice compared with tumors from wild-type mice. COX inhibitors reduce lung cancer growth both in vivo and in vitro (4,8,36,37). We therefore propose that the protective effect of cPLA$_2$ gene ablation is mediated at least in part through impairment of tumor-associated prostaglandin production. Elevated levels of PGE$_2$ are associated with a number of types of cancer, including human lung cancer (38–40), and disruption of cell surface receptors for PGE$_2$ is protective against tumorigenesis (41). Conversely, PGI$_2$, which is also produced downstream from COX, inhibits formation of tumors in mice, indicating a complex role for prostaglandins (9). Our data demonstrate alterations in prostaglandin production in the tumors of cPLA$_2$ null mice.

cPLA$_2$ activity also provides AA for other pathways and protection from lung tumorigenesis by cPLA$_2$ deletion could be mediated through additional lipid mediators. Leukotriene pathway inhibitors reduce mouse lung tumor multiplicity (5,6,42) and leukotrienes regulate several inflammatory pathways, including chemotaxis (12); cPLA$_2$-deficient mice may also have impaired leukotriene production. PAF levels are higher in uninvolved human lung cancer tissue than in tumors, and PAF may play a paracrine role in tumor growth by regulating vascular endothelial growth factor (43). Production of PAF involves the sequential action of cPLA$_2$ and acetyltransferase. While cPLA$_2$ is not believed to be rate-limiting for PAF production (44), the decreased levels of lysophospholipid associated with loss of cPLA$_2$ should decrease PAF levels. cPLA$_2$ expression in wild-type mice is especially pronounced in macrophages adjacent to the tumor (Figure 3); and these cells may be a source of PAF. In mice lacking cPLA$_2$, nearby macrophages deficient in PAF would be less potent at promoting tumorigenesis. Finally, it should be noted that cPLA$_2$ also hydrolyzes linoleic acid from membrane phospholipids. Downstream metabolism of linoleic acid to products such as 13-hydroxyoctadecadienoic acid may also contribute to lung tumorigenesis, and production would presumably be impaired in cPLA$_2$-deficient mice.

cPLA$_2$ activity was high in Kras-transformed human mammary epithelial cells (45), and more cPLA$_2$ mRNA (46,47), protein (48) and enzymatic activity were present in human colorectal tumors as compared with normal mucosa. cPLA$_2$ expression and activity in human gastric tumors were unchanged compared with normal mucosa (49). Mice with mutations in the Apc tumor suppressor gene spontaneously develop intestinal polyps that model human familial adenomatous polyposis (50). After crossing cPLA$_2$ $^-/$– mice with Apc$^{min}$ (51) or Apc$^{D176E}$ knockout mice (52), the Apc$^{min}$/cPLA$_2$ $^-/$– mice developed fewer small intestinal polyps and Apc$^{D176E}$/cPLA$_2$ $^-/$– mice had smaller polyps. Hong et al. (51) detected no differences in PGE$_2$ production in normal intestinal tissue from Apc$^{min}$/cPLA$_2$ $^-$/ mice compared with heterozygous littermates. Our results suggest that pathogenesis of lung tumors in mice is similar to cancer pathogenesis in the small intestine, since Apc$^{min}$/cPLA$_2$ $^-$/ mice developed fewer small intestinal polyps giving cPLA$_2$ a pro-tumorigenic role.

In conclusion, our data support an important role for cPLA$_2$ in lung tumorigenesis that is at least partly dependent on prostaglandin production. Therapeutic effects of cPLA$_2$ inhibitors may additionally act on targets distinct from those inhibited by NSAIDs. Combination therapies of cPLA$_2$ and COX inhibitors may offer more robust chemopreventive and chemotherapeutic strategies.

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