Induction of Cytosolic Phospholipase A$_2$ by Oncogenic Ras in Human Non-small Cell Lung Cancer* 

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Mutations in Ras family members that confer oncogenic potential are frequently observed in specific human cancers. We report that human non-small cell lung cancer (NSCLC) lines that harbor oncogenic mutations in Ki-Ras (H460, A549, H2122) synthesized high levels of prostaglandin E$_2$ (PGE$_2$) compared with NSCLC lacking Ras mutations or non-transformed lung epithelial cells (BEAS-2B). This increased PGE$_2$ production was mediated by constitutively high expression of 85-kDa cytosolic phospholipase A$_2$ (cPLA$_2$) and cyclooxygenase 2 (COX-2). The increased expression of cPLA$_2$ protein was mediated through elevated mRNA levels and activation of the cPLA$_2$ promoter. Induction of cPLA$_2$ promoter activity was blocked by expression of dominant-negative forms of Ras. Inhibition of Ras by the farnesyltransferase inhibitor BZA-5B inhibited prostaglandin synthesis in H2122 cells by decreasing expression of both cPLA$_2$ and COX-2. Finally, inhibitors of eicosanoid synthesis blocked anchorage-independent growth of NSCLC lines exhibiting Ki-Ras mutations. These results identify cPLA$_2$ as a novel Ras-inducible regulator of eicosanoid synthesis that participates in cellular transformation.

Lung cancer is the leading cause of cancer death in the United States. As a group, NSCLC constitutes the majority of lung cancers and gain-of-function mutations in the ras genes are frequently found both in primary NSCLC tumors and established NSCLC cell lines that exhibit the adenocarcinoma histology (1–3). Furthermore, the presence of mutated ras genes in NSCLC is associated with a shortened patient survival (4). A large body of work has now established that oncogenic Ras proteins, such as those observed in NSCLC, persistently activate intracellular signaling pathways that regulate the transcription of genes required for cell growth and transformation (5). Significantly, disruption of the post-translational processing of the Ras polypeptides with farnesyltransferase inhibitors has established that the Ras proteins perform a dominant transforming role in NSCLC that harbor constitutively activated Ras polypeptides (6).

Enhanced release of arachidonic acid, the precursor for a host of eicosanoids that modulate diverse physiological and pathophysiological responses, has been associated with transformation of fibroblasts by oncogenic Ras (7, 8) and Src (9). Arachidonic acid can be metabolized to a variety of bioactive lipid mediators through the action of three families of enzymes: cyclooxygenases, lipooxygenases, and cytochrome P450 epoxygenases. Two forms of cyclooxygenase (COX-1 and COX-2) have been described that convert arachidonic acid to PGH$_2$, the common precursor of prostaglandins, prostacyclin, and thromboxanes (10). COX-1 is constitutively expressed in most cells. COX-2 levels are low in resting cells and markedly induced by mitogenic and inflammatory agents, as well as oncogenic transformation (10). Observations that up-regulation of COX-2 occurs in human colorectal adenomas and adenocarcinomas (11, 12), coupled with the findings that the COX inhibitors decrease the occurrence of these tumors and hyperplasias (13, 14), indicate that metabolites of arachidonic acid serve as important co-factors in the progression of human cancers.

As the resting level of unesterified arachidonic acid is normally low within cells, activation of phospholipase A$_2$ (PLA$_2$), which releases arachidonic acid from phospholipid stores, represents the rate-limiting step in the biosynthesis of different eicosanoids. Multiple forms of PLA$_2$ have been described. Among the forms of PLA$_2$ expressed in mammalian tissues, the intracellular forms, of which cPLA$_2$ is perhaps the best characterized, are thought to mediate hormone-regulated arachidonic acid release (15). This enzyme, which is specific for arachidonate-containing phospholipids, is acutely regulated through increases in intracellular Ca$^{2+}$ (16, 17) and protein phosphorylation (18, 19). In addition to post-translational regulation, induction of cPLA$_2$ expression has been reported in response to cytokines such as interleukin-1β (20) and TNF-α (21), as well as growth factors such as epidermal growth factor (22). The promoter for cPLA$_2$ has been isolated from both human (23) and rat (24) and contains a number of putative regulatory elements, including AP-1 sites, NF-κB, and glucocorticoid regulatory elements. While is likely that constitutively elevated eicosanoid production is mediated by increased PLA$_2$ activity as well as induction of metabolizing enzymes, regulation of cPLA$_2$ has not been well examined in tumor cells. In this study we have examined the mechanisms leading to increased eicosanoid production in a panel of cultured human NSCLC lines.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Transfection—*Non-small cell lung cancer cell lines (H2122, A549, H1334, H661, and H460) and SV40-immortalized lung epithelial cells (BEAS-2B) were obtained from the University of Colorado Health Sciences Center Cancer Center Tissue Culture Core and maintained in RPMI 1640 containing 10% fetal bovine serum. For determination of anchorage-independent growth, single-cell suspen-

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The abbreviations used are: NSCLC, non-small cell lung cancer; PLA$_2$, phospholipase A$_2$; PGE$_2$, prostaglandin E$_2$; COX, cyclooxygenase; HPLC, high performance liquid chromatography.
sions of the indicated NSCLC lines were prepared, and portions containing 10,000 cells were suspended in 1.5 ml of RPMI 1640 containing 10% fetal bovine serum and 0.3% nobel agar and layered over a base prepared in 35-mm diameter dishes of RPMI 1640, 10% fetal bovine serum, and 0.5% agarose supplemented with the various eicosanoid synthesis inhibitors at twice the indicated concentration. The dishes were incubated for 3–4 weeks at 37 °C in a humidified CO2 incubator, and colonies were visualized under a microscope and counted. For experiments using the farnesyl transferase inhibitor BZA-5B (Genen-tech, South San Francisco, CA), stock solutions were prepared as described previously (25). Cells were treated for 5 days with fresh additions of drug every second day. Control cells were comparably treated with vehicle alone.

PGE2 and PLA2 Determinations—For determinations of PGE2, cells were incubated for 30 min at 37 °C, and PGE2 released into the medium was determined using a radioimmunnoassay kit (Amersham Corp.), following the manufacturer’s protocol. For PLA2 activity determinations, cells were harvested and PLA2 activity determined in the high speed supernatants using exogenous [14C]arachidonoylphosphatidylcholine, as described previously (17). Results are expressed a picomoles/min/mg.

RESULTS AND DISCUSSION

Previous investigations by Boyd and co-workers (26, 27) detected elevated prostaglandin levels in primary NSCLC lung tumor tissues and noted the synthesis of the various prostaglandins, especially PGE2 in several cultured NSCLC lines. We initially analyzed arachidonic acid metabolites released from NSCLC lines by reverse phase HPLC. The major metabolite identified by HPLC was confirmed by mass spectroscopy to be PGE2, although several unidentified minor metabolites were also detected (data not shown). Analysis by radioimmunoassay of PGE2 in medium from the various NSCLC lines and the SV40-immortalized human bronchial epithelial cell line, BEAS-2B, revealed significant PGE2 release in NSCLC lines H460, A549, and H2122 compared with low or undetectable synthesis by H661, H1334, and BEAS-2B cells (Fig. 1A). Significantly, high expression of the COX-2 protein in H460, A549, and H2122 correlated with marked production of PGE2 (Fig. 1A, inset). By contrast, COX-1 and 12-lipoxygenase were similarly expressed among the various cell lines (data not shown).

While induction of COX-2 presumably accounts for the high levels of PGE2 production in these NSCLC lines, availability of unesterified arachidonic acid through the action of PLA2 represents the rate-limiting step in eicosanoid production. Analysis of PLA2 activity in high speed supernatants prepared from extracts of the same panel of NSCLC lines revealed an extraordinarily high level of PLA2 activity in NSCLC lines H460, A549, and H2122 relative to BEAS-2B cells or NSCLC lines H661 and H1334 (Fig. 1B). Activity assayed in the absence of Ca2+ was less than 10% than that observed in the presence of Ca2+, indicating Ca2+-dependent form(s) of the enzyme. Immunoblot analysis of extracts from the panel of cell lines using a specific cPLA2 antiserum revealed high expression of cPLA2 protein in A549, H460, and H2122 cells relative to the little or no cPLA2 detected in BEAS-2B, H2122, and H661 cells (Fig. 1B, inset). Fractionation of cell extracts by Mono Q anion exchange revealed a single peak of PLA2 activity that co-migrated with purified cPLA2 (data not shown). Thus, increased expression of cPLA2 protein accounts for the high specific activity of PLA2 detected in NSCLC lines H460, A549, and H2122.

Northern blotting revealed that levels of cPLA2 mRNA were markedly induced in both H2122 and A549 cells compared with BEAS-2B or H1334 (Fig. 2A), suggesting increased transcription. In fact, transient expression of a cPLA2 promoter/luciferase construct in the NSCLC lines revealed a result revealed in a 8–20-fold in luciferase activity in H2122 cells compared with either BEAS-2B or H1334, which express little or no cPLA2 (Fig. 2B). These data argue for transcriptional activation of the cPLA2 gene as the mechanism for cPLA2 induction.

Previous analysis of the status of Ras genes in established lung cancer cell lines demonstrated that A549, H460, and H2122, which we have shown to exhibit high expression of cPLA2 and COX-2, express Ki-Ras molecules with mutations that confer gain-of-function (3). In contrast, H661 and H1334 express wild-type Ki-Ras polypeptides (28). The correlation of expression of GT-Pase-deficient Ki-Ras with high expression of cPLA2 and COX-2 supports our hypothesis that mutated Ras stimulates a signaling pathway leading to the transcriptional induction of the cPLA2. To test this possibility, NSCLC were therefore transiently co-transfected with a dominant-negative Ras construct (N17Ras) along with the cPLA2 promoter-luciferase construct. Expression of N17Ras significantly reduced the induction of cPLA2 promoter activity in H2122 (Fig. 2B), supporting a role for persistent Ras signaling in cPLA2 induction.

The role of oncogenic Ras in mediating eicosanoid production in NSCLC was further examined using the farnesyltransferase inhibitor BZA-5B. This drug has been shown to reverse transformation by oncogenic Ras in various cell types including NSCLC (6, 25), presumably by interfering with post-translational modifications of the protein required for function. BZA-5B potently inhibited the growth of H2122 cells that express oncogenic Ki-Ras, but had only a slight effect on the growth of BEAS-2B or H1334 cells which do not (Fig. 3A), confirming an important role for Ras in the transformed

![Cell Line](http://www.jbc.org)
growth of this cell line. Constitutive PGE\textsubscript{2} production was decreased by ~60\% in H2122 exposed to 100 \textmu M BZA-5B (+ BZA-5B: 1.52 ± 0.3 pg/\mu g of protein; vehicle: 3.78 ± 0.3 pg/\mu g of protein; \(p < 0.05\)). This decrease was associated with decreased cPLA\textsubscript{2} specific activity (Fig. 3B) and COX-2 expression (Fig. 3C).

To test the contribution of constitutive PGE\textsubscript{2} production to the transformed growth of the NSCLC lines, the influence of COX inhibitors on the ability of NSCLC cells to grow in soft agar-containing medium was examined (Fig. 4). The data reveal that increasing concentrations of sulindac sulfide, an effective inhibitor of both COX-1 and COX-2 (29), significantly inhibited the anchorage-independent growth of the H460, A549, and H2122 NSCLC lines. Indomethacin, a preferential inhibitor of COX-1 (29), was much less effective. Attempts to test the inhibitory potential of sulindac sulfide on NSCLC lines H661 and H1334, which produce little or no PGE\textsubscript{2}, were complicated by the poor cloning efficiency (<0.01\%) of these NSCLC lines in the soft agar assay.\textsuperscript{2} Analysis of the growth of NSCLC cells attached to plastic dishes revealed a cytostatic, not cytotoxic, action of sulindac sulfide. Thus, these findings support the involvement of eicosanoids generated by the action of cPLA\textsubscript{2} and COX-2 in the anchorage-independent growth of NSCLC. Interestingly, nordihydroguaiaretic acid and cinnamyl-3,4-dihydroxy-\(-\)-cyanocinnamate, selective inhibitors of lipoxygenase family members (30, 31), also potently blocked anchorage-independent NSCLC growth (Fig. 4). Like sulindac sulfide, nordihydroguaiaretic acid and cinnamyl-3,4-dihydroxy-\(-\)-cyanocinnamate exerted cytostatic effects in NSCLC cells grown on tissue culture plastic.

Elevated eicosanoid production has been observed in a number of tumor cells and is likely to contribute to the transformed growth of these cells. Mutations in Ras family members that inhibit GTPase activity and confer oncogenic potential are frequently observed in various human cancers in addition to NSCLC, including colon carcinomas, pancreatic adenocarcinomas, follicular and undifferentiated carcinomas of the thyroid, and acute myeloid leukemias (1). Induction of COX-2 and pro-

\textsuperscript{2} L. E. Heasley, B. Price, and R. A. Nemenoff, unpublished observations.
with a farnesyltransferase inhibitor, reduces expression of both enzymes. Previous studies in which oncogenic Ras was introduced in cultured cells have also documented an increase in cPLA2 activity (7) and support our findings in NSCLC. We have shown are representative of three independent experiments.

**Fig. 4.** Influence of eicosanoid synthesis inhibitors on the growth of NSCLC lines in soft agar-containing medium. Single-cell suspensions of the indicated NSCLC lines were analyzed for growth in soft agar as described under “Experimental Procedures.” The dishes were incubated for 3–4 weeks at 37 °C in a humidified CO2 incubator, and colonies were visualized under a microscope and counted. Results shown are representative of three independent experiments.

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