# Formation and antiproliferative effect of prostaglandin $E_3$ from eicosapentaenoic acid in human lung cancer cells

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**Abstract** **We investigated the formation and pharmacology of prostaglandin $E_3$ ($PGE_3$) derived from fish oil eicosapentaenoic acid (EPA) in human lung cancer A549 cells. Exposure of A549 cells to EPA resulted in the rapid formation and export of $PGE_3$. The extracellular ratio of $PGE_3$ to $PGE_2$ increased from 0.08 in control cells to 0.8 in cells exposed to EPA within 48 h. Incubation of EPA with cloned ovine or human recombinant cyclooxygenase 2 (COX-2) resulted in 13- and 18-fold greater formation of $PGE_3$, respectively, than that produced by COX-1. Exposure of A549 cells to 1 μM $PGE_3$ inhibited cell proliferation by 37.1% ($P < 0.05$). Exposure of normal human bronchial epithelial (NHBE) cells to $PGE_3$, however, had no effect. When A549 cells were exposed to EPA (25 μM) or a combination of EPA and celecoxib (a selective COX-2 inhibitor), the inhibitory effect of EPA on the growth of A549 cells was reversed by the presence of celecoxib (at both 5 and 10 μM). This effect appears to be associated with a 50% reduction of $PGE_3$ formation in cells treated with a combination of EPA and celecoxib compared with cells exposed to EPA alone. These data indicate that exposure of lung cancer cells to EPA results in a decrease in the COX-2-mediated formation of $PGE_2$, an increase in the level of $PGE_3$, and $PGE_3$-mediated inhibition of tumor cell proliferation.**—Yang, P., D. Chan, E. Felix, C. Cartwright, D. G. Menter, T. Madden, R. D. Klein, S. M. Fischer, and R. A. Newman. **Formation and antiproliferative effect of prostaglandin $E_3$ from eicosapentaenoic acid in human lung cancer cells. *J. Lipid Res.* 2004.** 45: **1030–1039.**

**Supplementary key words** ω-3 fatty acids • eicosanoid metabolism • cell proliferation • cyclooxygenase enzymes • malignant cells

Prostaglandins, which are generated by cyclooxygenase (COX) metabolism of arachidonic acid (AA), play a pivotal role in maintaining cell development and homeostasis of various diseases, especially inflammation and cancer [as reviewed in ref. (1)]. In contrast to a large body of evidence indicating that ω-6 polyunsaturated fatty acids such as AA promote the growth of tumor cells, ω-3 fatty acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] have actually been shown to inhibit tumor cell proliferation [as reviewed in ref. (2)]. Epidemiologic studies have shown an inverse relationship between blood levels of n-3 fatty acids derived from fish oils and the risk of prostate and lung cancers (3–5). However, molecular mechanisms for the pharmacologic anticancer activity of EPA have not been fully elucidated. A number of studies have suggested that the anticancer activities of both EPA and DHA are associated with their ability to inhibit the synthesis of 2-series prostaglandins, especially prostaglandin $E_2$ ($PGE_2$) production [as reviewed in ref. (6)]. In contrast to DHA, however, EPA can actually function as a substrate for COX and result in the synthesis of unique 3-series prostaglandin compounds (7). To date, studies reporting the formation of 3-series prostaglandins by EPA have been performed using normal cells or tissues (8, 9). Fischer and Weber (10), for example, provided the first evidence of in vivo formation of thromboxane $A_3$ and prostaglandin $I_3$ in humans fed fish oil. In addition, studies conducted in humans have shown that $PGE_3$ levels increased by ~10-fold in urine after ingestion of cod liver oil (40 ml/day) for 12 weeks (11).

In contrast to $PGE_2$, the biological activity of $PGE_3$ has received little attention. The effect of $PGE_3$ on cell growth has been reported only in normal murine mammary epithelial (12) and 3T3 fibroblast cells (13). Both studies showed that $PGE_2$ and $PGE_3$ stimulated the growth of nor-

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Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; calcein AM, acetoxymethyl ester of calcein; COX, cyclooxygenase; DAPI, 4′,6-diamidino-2-phenylindole; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC/MS/MS, liquid chromatography/tandem mass spectrometry; NHBE, normal human bronchial epithelial; $PGE_3$, prostaglandin $E_3$.

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mal cells but that $PGE_3$ was much less potent than $PGE_2$. A more recent study has suggested that $PGE_3$, unlike $PGE_2$, is not mitogenic to 3T3 fibroblasts (13). To date, however, the ability of cancer cells or even purified COX enzymes to form $PGE_3$ from EPA has not been evaluated. Furthermore, the biochemical pharmacology of $PGE_3$ in human cancer cells has not been investigated. In consideration of the documented higher level of expression of COX-2 in tumor cells than in normal cells (14), we sought to investigate *a*) the capability of human lung cancer A549 cells as well as cloned COXs to form $PGE_3$ using EPA as a substrate, *b*) the relative effects of $PGE_2$ and $PGE_3$ on human lung cancer cell proliferation, and *c*) the possible involvement of $PGE_3$ as an important factor in EPA-mediated changes in tumor cell growth.

In this study, we found that the level of $PGE_3$ was significantly increased after exposing A549 cells to EPA. The ability of cloned human recombinant COX-2 enzyme to form $PGE_3$ from EPA was much greater than that of COX-1 enzyme. In contrast to $PGE_2$, $PGE_3$ inhibited the growth of human lung tumor cells but not normal human bronchial epithelial (NHBE) cells. In addition, $PGE_3$ antagonized the effect of $PGE_2$ on cancer cell growth. Furthermore, the inhibitory effect of EPA on the growth of A549 cells was partially blocked by celecoxib (5 and 10 μM), and this effect was correlated with a reduced formation of $PGE_3$ in these cells.

## MATERIALS AND METHODS

### Reagents

$PGE_2$, $PGE_3$, $PGE_2$-$d_4$, anti-COX-1, and anti-COX-2 antibodies were obtained from Cayman Chemical Co. (Ann Arbor, MI). Acetoxymethyl ester of calcein (calcein AM) and 4′,6-diamidino-2-phenylindole (DAPI) fluorescent dyes were purchased from Molecular Probes (Eugene, OR). $PGE_2$ and $PGE_3$ were dissolved in ethanol/DMSO (1:1, v/v) and added directly to cell culture at a 1:1,000 ratio. We purchased methyl-[$^3$H]thymidine (specific activity, 6.7 Ci/mmol) from Perkin-Elmer (Boston, MA). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were HPLC grade (Fisher Scientific Co., Fair Lawn, NJ).

### Cell cultures

Human nonsmall cell lung cancer A549 cells (ATCC, Rockville, MD) and NHBE cells (Clonetics Corp., San Diego, CA) were used in this study. A549 cells were cultured in DMEM-F12

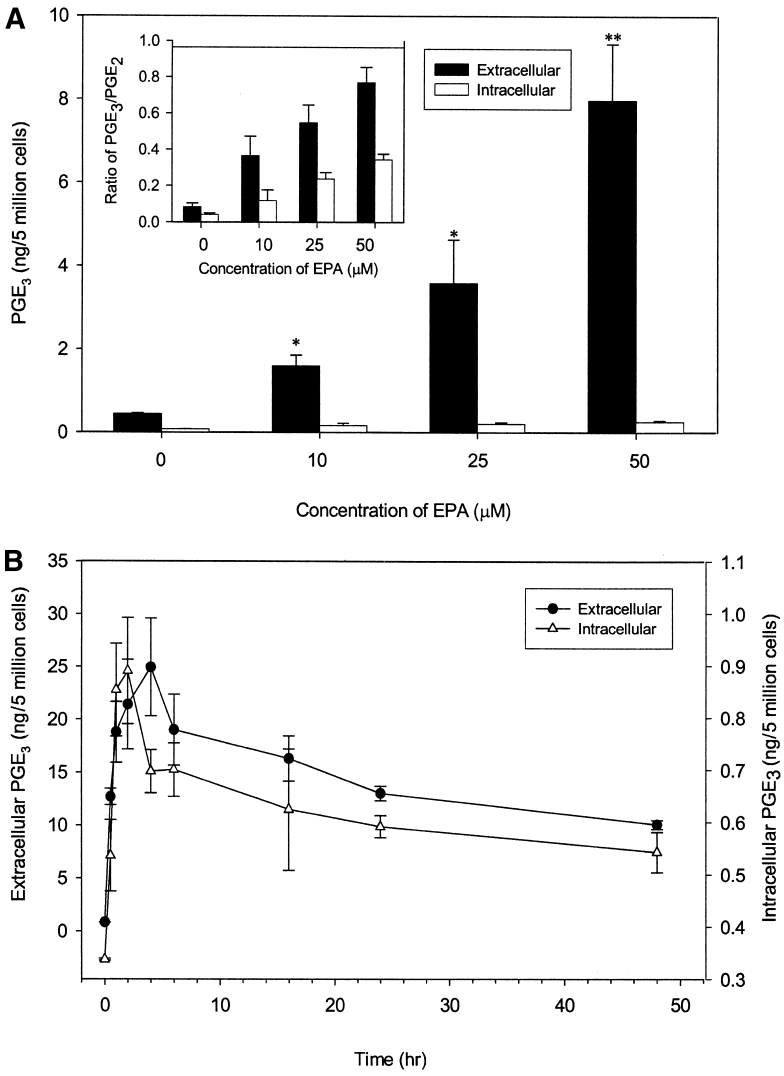


**Fig. 1.** Formation of prostaglandin $E_3$ ($PGE_3$) in A549 human lung cancer cells. Cells ($1 \times 10^6$) were exposed to 10–50 μM eicosapentaenoic acid (EPA) (A) or 50 μM EPA (B) in fresh serum-free medium containing 15 μM BSA before being harvested as described in Materials and Methods. Prostaglandins were extracted and analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS). A549 cells produced and exported $PGE_3$ formed from EPA in a concentration- and time-dependent manner. The inset in A shows the ratio of $PGE_3$ to $PGE_2$ within A549 cells treated with EPA. Data are presented as means ± SD of three separate experiments. * $P \leq 0.01$ and ** $P \leq 0.001$ versus control.

medium (Gibco BRL, Carlsbad, CA) supplemented with 5% heat-inactivated FBS (Hyclone Laboratories, Logan, UT), 2-mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). NHBE cells were grown in bronchial epithelial cell growth medium (Clonetics Corp.).

### $PGE_2$ and $PGE_3$ synthesis

A549 cells ($1 \times 10^6$) were plated in 100 mm culture dishes in complete DMEM-F12 medium and were allowed to attach overnight. Cells were then washed with PBS and treated with EPA (10–50 μM), AA (25–50 μM), or EPA plus AA (50 μM each) in fresh serum-free medium supplemented with 15 μM BSA for 24 or 48 h. To determine the time-dependent formation of $PGE_3$ from EPA, cells were exposed to 50 μM EPA and incubated for 1, 2, 4, 6, 16, 24, and 48 h. The culture medium was then collected, and cells were harvested by trypsinization and subjected to prostaglandin extraction.

The production of $PGE_2$ or $PGE_3$ by cloned COX-1 (ovine) or COX-2 (ovine or human recombinant) enzymes (Cayman Chemical Co.) was measured by incubation of 10 or 50 μM AA or EPA, respectively, with enzymes (15 U) in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 2 mM phenol, and 1 μM hematin. The reaction was stopped by the addition of 1 N citric acid before extraction of prostaglandins.

### Prostaglandin extraction and analysis

Intracellular prostaglandins were extracted according to the method of Yang et al. (15). Briefly, cell suspensions were washed with 2 ml of PBS and resuspended in 0.5 ml of PBS. Aliquots (40 μl) of 1 N citric acid, 5 μl of 10% butylated hydroxytoluene (BHT), and 20 μl of $PGE_2$-$d_4$ (100 ng/ml) as an internal standard were added to the cell suspensions or enzymatic reaction mixtures. Prostaglandins were extracted with 2 ml of hexane-ethyl acetate (1:1, v/v) three times. The upper organic phases were pooled and evaporated to dryness under a stream of nitrogen at room temperature. All extraction procedures were performed under conditions of minimal light. Samples were then reconstituted in 200 μl of methanol/10 mM ammonium acetate buffer (70:30, v/v), pH 8.5, before analysis by liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Prostaglandins in the cell culture medium were extracted using a solid-phase method. Aliquots of 10 μl of 10% BHT and 20 μl of $PGE_2$-$d_4$ were added to 1 ml of cell culture medium. The solution was applied to a Sep-Pak C18 cartridge (Waters Corp., Milford, MA) that had been preconditioned with methanol and water. Prostaglandins were eluted with 1 ml of methanol. The eluate was evaporated under a stream of nitrogen, and the residue was dissolved in 200 μl of methanol/10 mM ammonium acetate buffer (70:30, v/v), pH 8.5.

The extracted prostaglandins were quantitated by the LC/MS/MS method described by Yang et al. (15). Briefly, LC/MS/MS was performed using a Quattro Ultima tandem mass spectrometer (Waters Corp., Milford, MA) equipped with an Agilent HP 1100 binary pump HPLC inlet (Agilent Technologies, Palo Alto, CA). The prostaglandins were separated using a 2 × 150 mm Luna 3 μ phenyl-hexyl analytical column (Phenomenex, Torrance, CA). The mobile phase consisted of 10 mM ammonium acetate, pH 8.5, and methanol. The column temperature

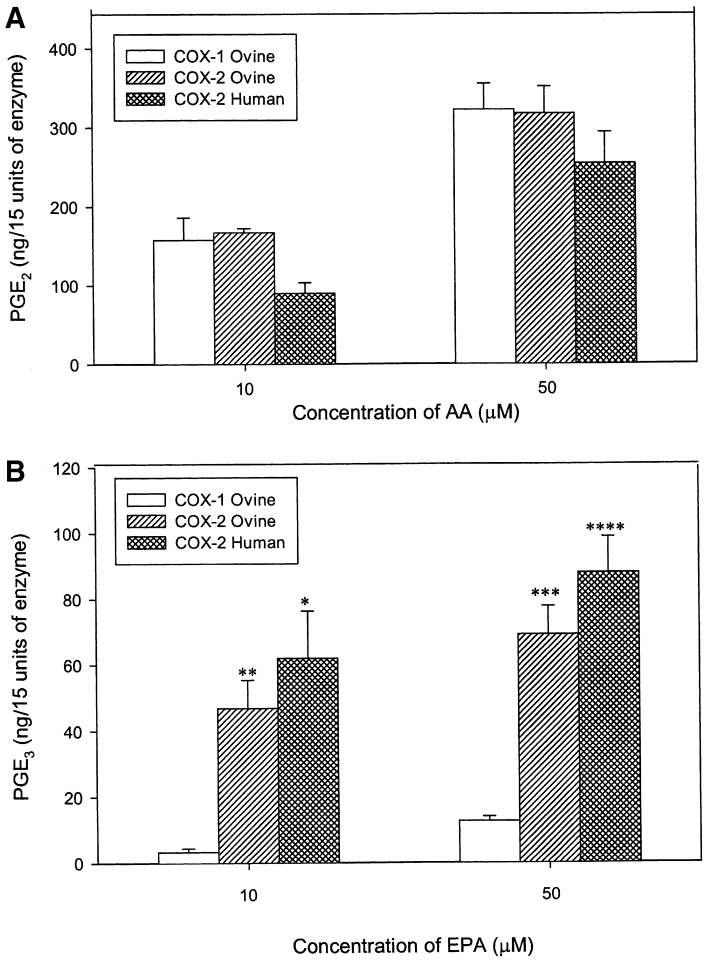


**Fig. 2.** Formation of $PGE_2$ [A; arachidonic acid (AA) as substrate] or $PGE_3$ (B; EPA as substrate) by cloned cyclooxygenase (COX) enzymes. COX-1 (ovine) and COX-2 (ovine or human recombinant; 15 U) were incubated with AA or EPA at the indicated concentrations for 5 min at 37°C. The reaction was terminated by adding 1 N citric acid and 0.1% butylated hydroxytoluene. Prostaglandins were extracted and analyzed by LC/MS/MS. Both COX-1 and COX-2 have a similar capacity to use AA as a substrate to produce $PGE_2$. The formation of $PGE_3$ by EPA, however, was at least three times greater with COX-2 than with COX-1. Data are presented as means ± SD of three separate experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$, and **** $P \leq 0.001$ versus COX-1.

was maintained at 50°C, and samples were kept at 4°C during the analysis. Individual analytes were detected using electrospray negative ionization and multiple reaction monitoring monitoring of the transitions $m/z$ 351 → 271 for $PGE_2$, $m/z$ 349 → 269 for $PGE_3$, and $m/z$ 355 → 275 for $PGE_2$-$d_4$. Fragmentation of all compounds was performed using argon as the collision gas at a collision cell pressure of $2.10 \times 10^{-3}$ Torr.

### Cell proliferation assay

The effects of $PGE_2$ and $PGE_3$ on cell proliferation were studied by determining the incorporation of [$^3$H]thymidine into cellular DNA (16). A549 and NHBE cells were grown in their complete medium for 24 h in 24-well culture plates to attain 70–75% confluence. Cells were then serum starved for 24 h before their use in experiments. Three hours before the termination of each experiment, 1 μCi of methyl-[$^3$H]thymidine was added to each well. After incubation, cells were washed twice with PBS, lysed with 0.5 N NaOH, and neutralized with 0.5 N HCl, and then radioactivity was measured.

### Western blotting

COX-1 and COX-2 protein expression in cells treated with EPA or AA was assessed by Western blotting. Cells were harvested using protein lysis buffer (50 mM Tris-HCl, 250 mM NaCl, 0.1% NP-40, 25 μg/ml leupeptin, and 0.5 mM PMSF). The protein concentration in the supernatant fraction was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 100 μg of protein was resuspended in 20 μl of Laemmli sample buffer (Bio-Rad), boiled for 5 min at 95°C, and run on a 7.5% SDS-PAGE gel before being subjected to Western blotting. COX-2 purified protein (Cayman Chemical Co.) was used as the electrophoresis standard. Proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad) and subjected to immunoblot analysis. Membranes were blocked overnight at 4°C in 5% skim milk powder diluted in TBS. Thereafter, membranes were incubated with either COX-1 or COX-2 antibodies (1:2,000) (Cayman Chemical Co.) for 2.5 h at room temperature. After transfer, membranes were incubated for 1 h with goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membranes were developed by the enhanced chemiluminescence system (Amersham Bioscience, Buckinghamshire, UK) and exposed to Kodak XAR-5™ film.

### Cell viability and DNA staining

Cell viability was determined using various treatments or combinations of EPA, celecoxib, or SC-560. The assay was performed using the vital dye calcein AM (CAM) ester (Molecular Probes), which is plasma membrane permeable and nonfluorescent before activation by nonspecific esterases within viable cells. The cleavage product CAM emits green fluorescence that is retained by viable cells and cells in the early stages of apoptosis. When plasma membrane integrity is disrupted in the latter stages of cell death, cells fail to effectively retain CAM. Cells were plated in 96-well plates and incubated with 25 μM EPA or AA, SC-560 (0.1 and 0.5 μM), and celecoxib (5 and 10 μM) and various combinations of EPA and SC-560 or celecoxib for 24 h. Cells then were incubated with CAM ester (1 μM), DAPI (300 nM), and propium iodide (PI) (500 nM) (Molecular Probes) in HEPES-buffered saline solution for 15 min at 25°C. Nuclear morphology, DNA dye uptake, and cellular staining were assessed by fluorescence microscopy using an IPLabs image-analysis system (Scanalytics, Inc., Fairfax, VA) on an IX70 inverted microscope (Olympus, Melville, NY) with a Quantix charge-coupled device camera (Roper Scientific, Trenton, NJ). Fluorescence was quantitated using a Biolumin 9600 well plate reader. Cells lysed with 1% NP-40 detergent were used as a negative control for CAM ester conversion and as a positive control for DNA intercalating dye uptake. Cells used as a viable cell-positive control had intact plasma membranes and converted CAM ester to fluorescent CAM in a linear manner. They completely excluded DAPI and PI uptake.

### Statistical analysis

Student's $t$-test was used to determine the statistical differences between various experimental groups; a value of $P < 0.05$ was considered significant.

## RESULTS

### Formation of $PGE_3$

The anticancer effect of EPA has been linked to the inhibition of COX activity and consequently reduced levels of $PGE_2$ both in vitro and in vivo (17, 18). We have previ-

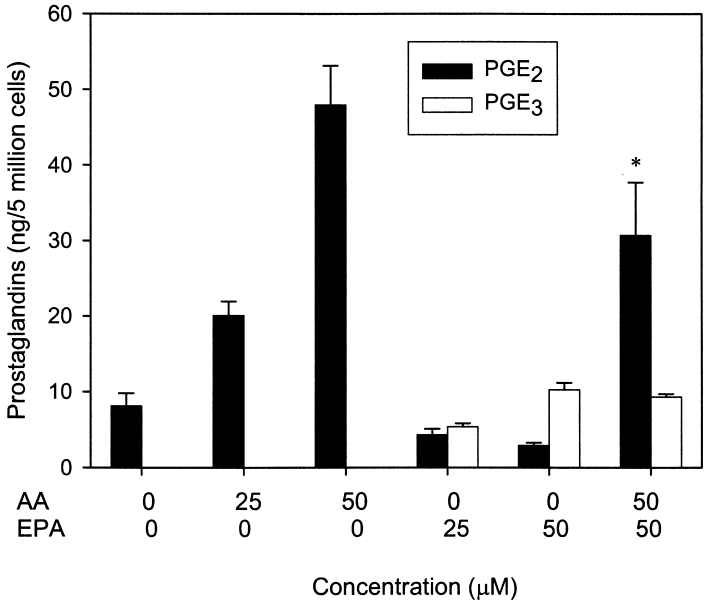


**Fig. 3.** Effects of EPA on the activity of COX-2 enzyme in A549 cells. A549 cells ($1 \times 10^6$) were grown in fresh serum-free medium containing 15 μM BSA and then treated with AA (25 and 50 μM) or EPA (25 and 50 μM) alone or with AA and EPA (50 μM plus 50 μM). After 24 h, the culture medium was collected and prostaglandins were extracted and analyzed by LC/MS/MS. In cells treated with both AA and EPA, the level of $PGE_2$ was significantly reduced compared with that in cells treated with AA alone. The concentration of $PGE_3$, however, remained the same as that in cells treated with EPA alone. Data are presented as means ± SD of duplicate samples from three separate experiments. * $P \leq 0.05$ versus treatment with AA alone.

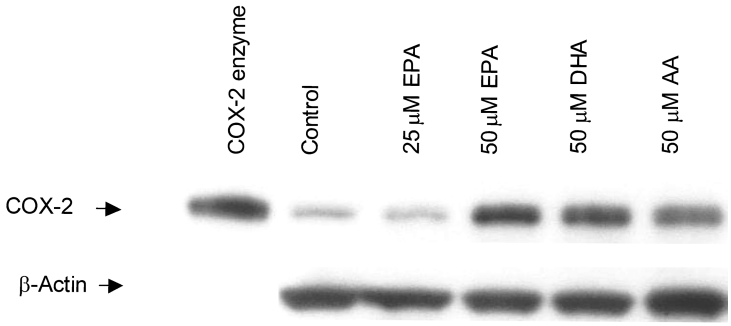


**Fig. 4.** Effect of EPA on the expression of COX-2 in A549 cells. Cells ($2 \times 10^6$) were grown in medium supplemented with 15 μM BSA alone or in medium containing EPA (25 and 50 μM), docosahexaenoic acid (DHA; 50 μM), and AA (50 μM) for 24 h. COX-2 protein expression was determined with Western blotting as described in Materials and Methods. All three fatty acids tested induced COX-2 expression.

ously shown that a clinically achievable plasma level of EPA (100 μM) (19) results in more than a 50% reduction in $PGE_2$ formation in lung cancer cells (15). Because EPA is a substrate for COX (7), we determined the capacity of lung cancer cells to form $PGE_3$ from the ω-3 fatty acid EPA. When A549 cells were exposed to EPA (10 and 50 μM) for 48 h, the concentration of $PGE_3$ in the cell culture medium significantly increased to as high as 7.98 ± 1.36 ng/5 million cells compared with that in the control group (0.44 ± 0.021 ng/5 million cells; $P < 0.001$) (**Fig. 1A**). This increase of extracellular $PGE_3$ was concentration dependent. When A549 cells were exposed to 50 μM EPA for 48 h, the extracellular ratio of $PGE_3$ to $PGE_2$ increased from 0.08 in control cells to 0.8 in treated cells (Fig. 1A, inset). The increase in the ratio of $PGE_3$ over $PGE_2$ was also concentration dependent.

As shown in Fig. 1B, exposure of A549 cells to 50 μM EPA for 30 min resulted in a rapid ~13-fold increase in extracellular $PGE_3$ level, from 0.85 ± 0.12 ng/5 million cells in control cells to 12.68 ± 0.77 ng/5 million cells in treated cells. After incubation with EPA for 4 h, the intracellular level of $PGE_3$ increased from 0.33 ± 0.021 ng/5 million cells in control cells to 0.88 ± 0.11 ng/5 million cells in treated cells, whereas the extracellular level of $PGE_3$ reached 24.94 ± 4.62 ng/5 million cells. These data suggest that A549 human lung cancer cells are able to rapidly produce and export high amounts of $PGE_3$. The extracellular peak level of $PGE_3$ occurred at 6 h, after which it declined; by 48 h, levels of $PGE_3$ had decreased by at least 50% from the peak concentration (Fig. 1B).

We next asked whether the production of $PGE_3$ in A549 cells was the result of COX-1, COX-2, or both enzymes (A549 cells express both COX-1 and COX-2 protein) (20). Cloned ovine COX-1 and human recombinant COX-2 were used to test for the formation of $PGE_2$ or $PGE_3$ using AA and EPA, respectively, as substrates. As shown in **Fig. 2A**, both COX-1 and COX-2 enzymes produced $PGE_2$ with comparable efficiency using 10 μM AA as a substrate, a finding previously reported by other investigators (21, 22). In contrast, the formation of $PGE_3$ from EPA was at least three times greater with COX-2 than with COX-1 (Fig. 2B). With 10 μM EPA, the relative efficiency of the enzymes using this substrate to form $PGE_3$ was human recombinant COX-2 (61.9 ± 14.2 ng/15 U enzymes) > ovine COX-2 (46.9 ± 8.5 ng/15 U enzymes) > ovine COX-1 (3.4 ± 1.0 ng/15 U enzymes). Overall, the total production of $PGE_2$ was 15- and 3-fold higher with both COX-1 and COX-2 enzymes, respectively, than the total production of $PGE_3$ by these enzymes.

The relative ability of A549 cells to form $PGE_2$ from AA while simultaneously being exposed to EPA was then de-

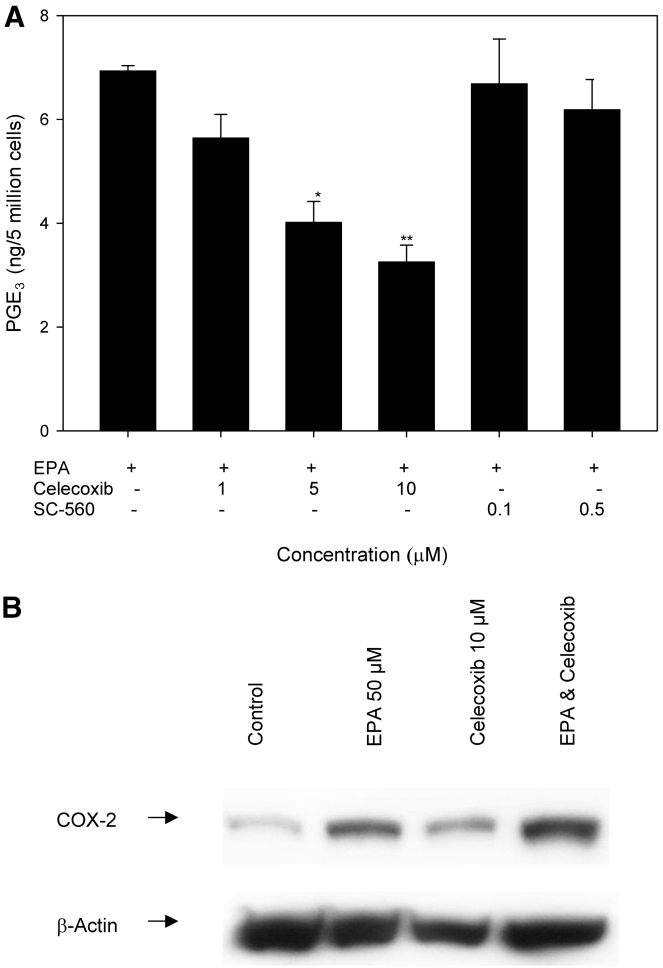


**Fig. 5.** Effect of celecoxib on the formation of $PGE_3$ (A) and the expression of COX-2 (B) in A549 cells. A: Cells ($2 \times 10^5$) were plated in six-well plates overnight and then treated with EPA (25 μM) and celecoxib (5 and 10 μM) or EPA and SC-560 (0.1 and 0.5 μM) for 24 h. The eicosanoids in the medium were extracted and analyzed as described in the text. Celecoxib inhibited the formation of $PGE_3$ in a concentration-dependent manner; however, SC-560 did not alter the levels of $PGE_3$ mediated by EPA. Data are presented as means ± SD from three separate experiments. * $P < 0.05$ and * * $P < 0.001$ versus EPA-treated cells. B: Both EPA and a combination of EPA and celecoxib dramatically induced COX-2 expression in A549 cells.

termined. The level of $PGE_2$ in cells treated with both AA and EPA (50 μM each) decreased by 35% in comparison with that in cells treated with AA alone (50 μM). It was interesting that the formation of $PGE_3$ was similar in cells treated either with EPA alone (50 μM) or with AA plus EPA (50 μM each) (**Fig. 3**).

### EPA and COX-2 protein expression

Because the levels of $PGE_3$ increased rapidly after A549 cells were exposed to EPA, the data suggested to us that the expression of COX-2 protein might be induced. Therefore, we examined the relative abilities of EPA and AA to induce COX-2 protein. A549 cells were exposed to 50 μM EPA for 2, 4, 6, 16, and 24 h, and induction of COX-2 protein was determined by Western blot analysis. Unexpectedly, the expression of COX-2 protein was not increased until 16 h. **Figure 4** shows that 24 h of exposure of cells to EPA (25 and 50 μM) induced COX-2 protein expression in A549 cells in a concentration-dependent manner. Interestingly, DHA and AA (50 μM) also induced the expression of COX-2 protein in A549 cells. Expression of COX-1 protein, however, was not altered (data not shown).

### Inhibition of $PGE_3$ formation by celecoxib but not SC-560

To further confirm that $PGE_3$ was enzymatically generated either by cloned enzymes or by cancer cells, the formation of $PGE_3$ by EPA in the presence of either celecoxib, a selective COX-2 inhibitor, or SC-560, a selective COX-1 inhibitor, in A549 cells was tested. Celecoxib (5 and 10 μM) significantly reduced $PGE_3$ levels to 4.25 ± 0.40 and 3.25 ± 0.35 ng/5 million cells, respectively, from the control cell level of 6.92 ± 0.24 ng/5 million cells ($P < 0.05$). The inhibition of $PGE_3$ formation by celecoxib in A549 cells was concentration dependent. In comparison, SC-560 up to 0.5 μM, a concentration known to inhibit 95% of COX-1 activity (23), had no effect on the production of $PGE_3$ mediated by EPA (**Fig. 5A**). We then tested the inhibitory effect of celecoxib on $PGE_3$ formation with cloned COX-2 enzymes and found that celecoxib (10 μM) also inhibited the production of $PGE_3$ by EPA by ~72% (data not shown). The expression of COX-2 protein in EPA-treated cells in the presence of celecoxib was also examined. Interestingly, when cells were exposed to both EPA and celecoxib, the presence of celecoxib did not alter the expression of COX-2 protein compared with that in cells treated with EPA alone (Fig. 5B).

### $PGE_3$ and cancer cell proliferation

Studies have suggested that $PGE_2$ can stimulate colorectal cancer cell growth (16); thus, the effect of both $PGE_2$ and $PGE_3$ on human lung cancer cell proliferation was investigated. After A549 cells were exposed to either $PGE_2$ or $PGE_3$ for 24 h, $PGE_2$ (1 μM) produced only a slight increase in cell proliferation, whereas $PGE_3$ (1 μM) inhibited the growth of these cells by 37.1% ($P < 0.05$) (**Fig. 6**). Addition of $PGE_3$ to the $PGE_2$-treated A549 cells also led to a significant reduction in cell proliferation compared with that in the cells treated with $PGE_2$ alone ($P < 0.05$). The effect of $PGE_3$ on A549 cell proliferation was concentration dependent. In contrast to that of A549 cells, expo-

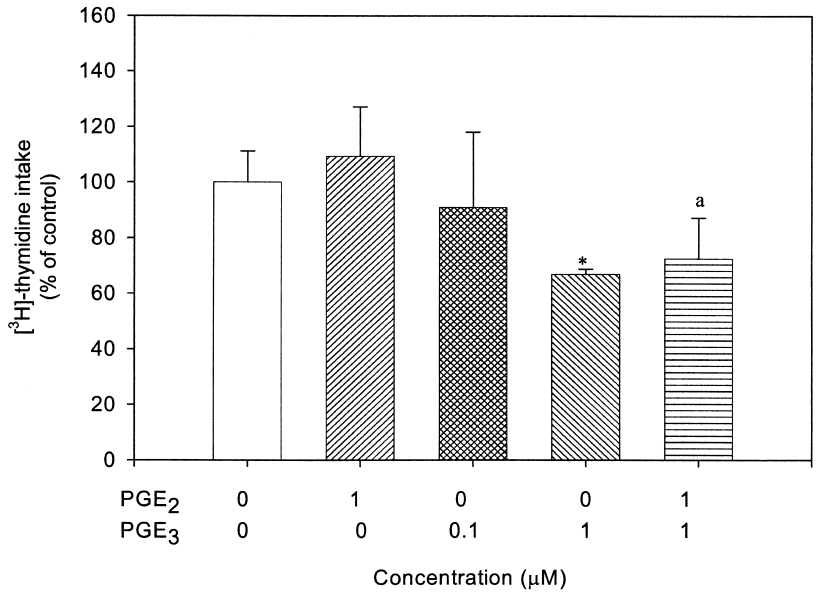


**Fig. 6.** Effect of $PGE_2$ and $PGE_3$ on the proliferation of A549 cells. Cell growth was synchronized using serum starvation. Cells were then treated with $PGE_2$, $PGE_3$, or a combination of $PGE_2$ and $PGE_3$ at the indicated concentrations for 24 h. Three hours before the end of experiment, 1 μCi of methyl-[$^3$H]thymidine was added, and incorporation of the radioactivity into DNA was measured. $PGE_3$ (1 μM) significantly inhibited the proliferation of A549 cells. Addition of $PGE_3$ to $PGE_2$-treated cells blocked the $PGE_2$-mediated proliferation of A549 lung cancer cells. Data are presented as means ± SD of three separate experiments. [a] $P \leq 0.05$ versus treatment with $PGE_2$ alone, * $P \leq 0.05$ versus control.

sure of NHBE cells to 1 μM $PGE_3$ reduced the DNA synthesis of these cells by only 10%.

### Celecoxib reversed EPA-induced cell death

The role of $PGE_3$ in EPA-induced cell death was further examined in A549 cells treated with EPA alone and a combination of EPA and selective COX inhibitors. A549 cells were exposed to EPA (25 μM), AA (25 μM), EPA and celecoxib (5 and 10 μM), and EPA and SC-560 (0.1 and 0.5 μM) for 24 h. Cell viability and apoptosis were detected by staining the cells with CAM and DAPI. Nuclear morphology was also recorded with a fluorescence microscope. When cells were exposed to EPA (25 μM) for 24 h, the majority of cells were rounded up and appeared to be undergoing apoptosis, as detected by staining with DAPI dye (**Fig. 7**, upper panel). Interestingly, when the cells were exposed to the combination of EPA and celecoxib (Fig. 7, middle panel), cell death was reduced dramatically, as indicated by the increased viability of cells detected by calcein AM staining (green fluorescence) compared with treatment with EPA alone. In contrast, SC-560 only slightly affected EPA-induced cell death (Fig. 7, lower panel). Cell viability and apoptosis were additionally quantitated using a Biolumin 9600 well plate reader. Exposure of cells to 25 μM EPA led to a 63% reduction in cell viability ($P < 0.05$) and increased apoptotic cells by 5-fold (**Fig. 8**) ($P < 0.001$) compared with control cells. In contrast, exposure of cells to a similar concentration of AA did not affect the growth of A549 cells. Furthermore, when cells were exposed to EPA and celecoxib (5 and 10 μM), the number of apoptotic cells was significantly reduced by 38% and 56%, respectively, compared with treatment with EPA alone. The reversible effect of celecoxib on EPA-induced cell growth inhibition was concentration dependent. Interestingly, SC-560, a selective COX-1 inhibitor, did not block the inhibitory effect of EPA on A549 cell growth (Fig. 8). Taken together, these data suggest that the inhibitory effect of EPA on the growth of A549 cells might be at least partially associated with the formation of $PGE_3$.

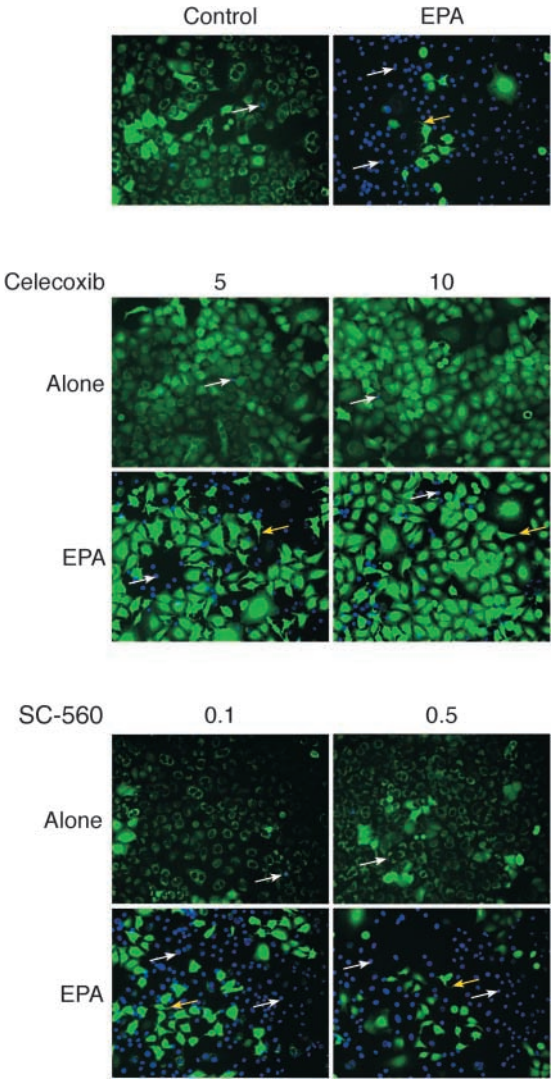


**Fig. 7.** Fluorescence microscopic examination of A549 cells. A549 cells ($1 \times 10^4$) were plated in a 96-well plate overnight in DMEM-F12 medium. Cells were then treated with fresh serum-free medium with 15 μM BSA containing 25 μM EPA, celecoxib (5 and 10 μM), SC-560 (0.1 and 0.5 μM), and combinations of EPA and the two inhibitors for 24 h. Fluorescence cocktail including calcein AM (CAM) ester, 4′,6-diamidino-2-phenylindole (DAPI), and propium iodide was added and incubated for 15 min at room temperature as indicated in Materials and Methods. CAM ester staining of the esterase in viable cells showed as green fluorescence, and DAPI-counterstained DNA in nuclei showed as blue. There was a remarkable amount of blue staining in the cells treated with EPA alone in comparison with the control cells. However, much more CAM staining appeared in the cells treated with celecoxib and EPA compared with EPA alone.

## DISCUSSION

Studies have suggested that altered production of prostaglandins might be associated with the beneficial effects of the consumption of ω-3 fatty acids (24). In contrast to data from numerous studies with $PGE_2$, little is known of the pharmacology of and cellular response to $PGE_3$. Quantitation of $PGE_3$ in biological matrices as well as within cell culture has been difficult, and this has hampered studies of the relative formation of this unique prostaglandin and its effects on the formation of $PGE_2$ within normal and malignant cell populations.

By applying a selective and sensitive LC/MS/MS method for the determination of $PGE_3$, we previously showed that $PGE_3$ is formed within 5 min of exposure of A549 cells to EPA (15). In the present study, we have demonstrated for the first time that both human lung cancer cells and cloned ovine COX-1 and COX-2 and human recombinant COX-2 enzymes are capable of generating $PGE_3$ from EPA in a concentration- and time-dependent manner. In addition, we also found that $PGE_3$ inhibited the proliferation of A549 human lung cancer cells but did not alter the growth of normal NHBE cells. Furthermore, addition of $PGE_3$ to $PGE_2$-treated cells resulted in the inhibition of cell proliferation in A549 cells. Our data also demonstrate that celecoxib can partially block EPA-induced cell death,

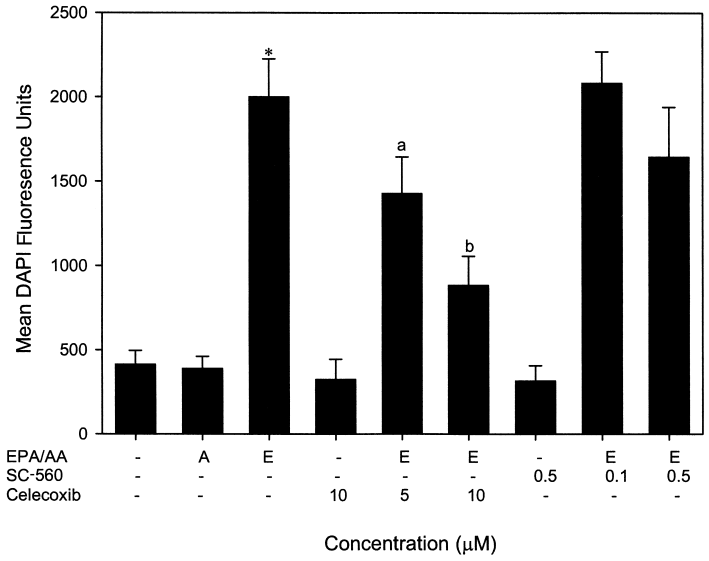


**Fig. 8.** DNA staining on A549 cells. Cells ($1 \times 10^4$) were cultured in DMEM-F12 medium with 5% FBS. Cells were then placed in fresh serum-free medium containing 15 μM BSA and treated with EPA or AA (25 μM), SC-560, and celecoxib at conditions described in Materials and Methods. After 24 h, fluorescence cocktail was added and fluorescence was quantitated on a Biolumin 9600 plate reader as described in Materials and Methods. EPA led to significant increases in the apoptotic cells. When cells were exposed to EPA and celecoxib, cell death induced by EPA was reversed by celecoxib, as indicated by a 50% reduction in apoptotic cells compared with EPA alone. Values are averages of two separate experiments with eight duplicates from each experiment (means ± SD). * $P <$ 0.001 (significantly different from untreated cells); [a] $P < 0.05$ and [b] $P < 0.01$ versus EPA-treated alone.

which, in turn, is associated with a reduced formation of $PGE_3$ in A549 cells.

Increased expression of COX-2 protein and the consequent production of $PGE_2$ have been implicated in the pathogenesis of several types of cancer, including colon, breast, and lung (25). In this study, we used A549 human lung cancer cells to examine the AA-mediated formation of $PGE_2$ as well as the formation of $PGE_3$ from EPA. Exposure of A549 cells to EPA led to an increase in intracellular $PGE_3$ levels that peaked 2 h after the addition of substrate. It is clear that rapid transport of this 3-series prostaglandin occurred, which led to the maximal level of $PGE_3$ at 4 h in the culture medium. Both the intracellular and extracellular levels of $PGE_3$, however, were reduced at 6 h. One explanation for this phenomenon is that $PGE_3$ may be unstable at 37°C. To test this hypothesis, $PGE_2$ and $PGE_3$ were incubated with DMEM-F12 medium only (i.e., without FBS or cells present). The levels of both prostaglandins declined after 6 h of incubation (data not shown), suggesting that thermal degradation of the prostaglandins had occurred. Additionally, $PGE_3$ may be further metabolized by prostaglandin dehydrogenase (26). Low basal or endogenous levels of $PGE_3$ in A549 cells were observed in cells that had not been exposed to EPA. The explanation for this lies in the fact that the FBS commonly added to our tissue culture medium contains a trace amount of EPA. We analyzed several lots of FBS sold for use in tissue culture and found low levels of EPA in FBS from two different commercial sources (data not shown). Although the low levels of EPA present in the FBS did not affect the findings of the reported studies, researchers who examine COX-related activity in cells in culture should be aware of EPA present in commercially supplied FBS.

In prior investigations of the relative ability of COX to form 3-series prostaglandins, microsomal preparations from sheep vesicular glands were used that may have contained a relatively high content of COX-1 as opposed to COX-2 (27). To clarify the enzyme-specific production of the products, we examined the formation of both $PGE_2$ and $PGE_3$ from cloned COX-1 and COX-2 enzymes. The production of $PGE_2$ by ovine COX-1 and human recombinant COX-2 exposed to AA was similar to that previously reported (21, 22). In contrast, the formation of $PGE_3$ with human recombinant COX-2 was 5- to 18-fold higher than that from COX-1 enzyme. These data are consistent with those reported by Laneuville et al. (21) demonstrating that the rates of oxygenation of EPA are three times greater with intact cell and microsomal preparations of COX-2 than they are with comparable preparations of COX-1. In contrast to findings from studies showing that EPA (compared with AA) is a poor substrate for COX-1 and COX-2, as measured by the rate of oxygen uptake (27), our data suggest that the COX-2-mediated formation of $PGE_3$ from EPA (61.91 ± 14.34 ng/15 U enzyme) was only slightly less than the formation of $PGE_2$ from AA (90.11 ± 13.42 ng/15 U enzyme) using human recombinant COX-2. Taken together, our data demonstrate that EPA is a better substrate for human COX-2 than it is for COX-1 enzyme.

It was previously shown that EPA and AA induce the expression of COX-2 protein and mRNA in keratinocytes (28) and macrophages (29). We also observed that EPA, DHA, and AA (50 μM) are all capable of inducing the expression of COX-2 protein in A549 cells in 24 h. In contrast to a previous study demonstrating that the induction of COX-2 expression in keratinocyte cells occurs within 3 h of treatment (28), we did not observe a significant induction of COX-2 expression by EPA in A549 cells until 16 h of treatment. This suggests that the rapid increase of $PGE_3$ in A549 cells (peaking at 4 h) may result from the metabolism of EPA by both COX-1 and COX-2 enzymes in the cells. Even though the levels of COX-2 protein were increased by exposure of A549 cells to EPA, the relative formation of $PGE_2$ by AA in those cells was actually reduced by exposure to EPA. The significance of the relative induction of COX-2 protein expression in the presence of COX-2 inhibitors, however, is unclear. For example, a number of in vivo studies have shown that ingestion of

fish oil supplement (containing both EPA and DHA) inhibited colon or breast MDA-MB-231 tumor growth and that this anticancer effect may have been associated with the inhibition of expression of COX-2 protein at the tumor site (30, 31). In this study, the addition of AA did not alter the formation of $PGE_3$ by EPA in A549 cells. Thus, even though the expression of COX-2 protein may increase within cells, the beneficial effects of EPA may involve both a decrease in the COX-2-mediated formation of $PGE_2$ and the unaltered formation of $PGE_3$, with its consequent effects on inhibition of tumor cell growth.

An important observation made in this study was the ability of $PGE_3$ to inhibit the proliferation of A549 human lung cancer cells. When A549 tumor lung cells were exposed to both prostaglandins, $PGE_2$ did not block the inhibitory effect of $PGE_3$ on the proliferation of these cells. More interestingly, celecoxib at 5 μM, a concentration known to produce at least a 50% reduction in $PGE_2$ formation in A549 cells (32), partially reversed EPA-induced cell death as indicated by DAPI staining. This observation correlates well with the reduction of $PGE_3$ formation in cells treated with EPA and celecoxib. These results indicate that the inhibitory effect of EPA on tumor cell proliferation is attributable in part to the formation of $PGE_3$, which actively functions as a suppressor of cell proliferation. Other mechanisms have been proposed to explain the cell growth inhibitory effect of EPA. Studies have indicated, for example, that dietary EPA and DHA can induce compositional changes in colonic mitochondrial membrane phospholipids that may, in turn, facilitate their inhibitory effect on cell proliferation as well as the induction of apoptosis in tumor cells (2, 33). To our knowledge, this is the first study to demonstrate that $PGE_3$ produces antiproliferative effects on human nonsmall cell lung cancer cells. In contrast to other reports, we did not observe a significant stimulatory effect of $PGE_2$ on the growth of either cancer or normal cells (12, 16). A potential explanation for this is that because the endogenous level of $PGE_2$ within A549 cells is already relatively high (~10 ng/5 million cells), the addition of exogenous $PGE_2$ may have been incapable of further stimulating the growth of these rapidly dividing tumor cells. An additional point is that the effect of $PGE_2$ on cell proliferation may be cell type specific. For example, $PGE_2$ can induce the stimulation of normal mammary epithelial cell or colon cancer cell growth (17, 34), yet it may have an antiproliferative effect on B16 melanoma cells or B lymphocytes (35). Nevertheless, the selectivity and specificity of the inhibitory effect of $PGE_3$ on the proliferation of tumor cells is of interest and warrants further investigation.

In summary, we have found that A549 human lung cancer cells can produce and promptly export $PGE_3$ using EPA as a substrate in a time- and concentration-dependent manner. Cloned ovine COX-1 and COX-2 and human recombinant COX-2 were all able to generate $PGE_3$ when incubated with EPA. However, the ability of COX-2 to form $PGE_3$ was much greater than that of COX-1. $PGE_3$, unlike $PGE_2$, inhibited the proliferation of human lung cancer cells and antagonized the effect of $PGE_2$ on cell proliferation in A549 human lung cancer cells. Our newly developed LC/MS/MS analytical method allowed us to clearly determine the formation of $PGE_3$ in both intact cells and cell-free systems. Therefore, the increased expression of COX-2 protein in cancer cells and the resulting increased production of $PGE_3$ from EPA may provide a novel mechanism for the anticancer activity of fish oil that warrants further investigation.

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