

Arachidonic acid, an omega-6 fatty acid, induces cytoplasmic phospholipase A₂ in prostate carcinoma cells

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For the past 60 years, dietary intake of essential fatty acids has increased. Moreover, the omega-6 fatty acids have recently been found to play an important role in regulation of gene expression. Proliferation of human prostate cells was significantly increased 48 h after arachidonic acid (AA) addition. We have analyzed initial uptake using Nile red fluorescence and we found that the albumin conjugated AA is endocytosed into the cells followed by the induction of RNA within minutes, protein and PGE₂ synthesis within hours. Here we describe that AA induces expression of cytosolic phospholipase A₂ (cPLA₂) in a dose-dependent manner and that this upregulation is dependent upon downstream synthesis of PGE₂. The upregulation of *cox-2* and *cPLA₂* was inhibited by flurbiprofen, a cyclooxygenase (COX) inhibitor, making this a second feed-forward enzyme in the eicosanoid pathway. Cox-2 specific inhibitors are known to inhibit colon and prostate cancer growth in humans; however, recent findings show that some of these have cardiovascular complications. Since cPLA₂ is upstream in the eicosanoid pathway, it may be a good alternative for a pharmaceutical target for the treatment of cancer.

Introduction

Lipid requirements of the mammal are met by dietary intake and *de novo* synthesis; the omega-6 fatty acids (ω -6 FAs) are essential since they cannot be synthesized by mammals and must be supplied in the diet. Linoleic acid (LA) and its product, arachidonic acid (AA) are transformed into prostaglandins (PG) and thromboxanes (TX) by the enzyme prostaglandin endoperoxide synthase (PES), also referred to as cyclooxygenase (COX; EC 1.14.99.1), or into leukotrienes (LT) by the enzyme lipoxygenase (1,2). Prostaglandins are short-lived and act in an autocrine or paracrine manner to convey their biological effects. Many nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, flurbiprofen and indomethacin, exert their effects by inhibiting synthesis of prostaglandins.

Abbreviations: ω -6 FAs, omega-6 fatty acids; AA, arachidonic acid; COX, cyclooxygenase; EIA, Enzyme immunoassay; LA, linoleic acid; LDLr, low-density lipoprotein receptor; LT, leukotrienes; NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandins; TX, thromboxanes.

Mammalian cells contain at least two isozymes of cyclooxygenase, COX-1, a well characterized, constitutively expressed enzyme and COX-2 which is inducible following the addition of a variety of growth-promoting stimuli (1–3). We have previously shown that the low-density lipoprotein receptor (LDLr) allows LDL to effectively deliver AA to prostate and colorectal tumor cells, consequently inducing COX-2 message and cell proliferation (4–7).

Intracellular levels of free AA are controlled by series of reactions in which the fatty acid is released from membrane phospholipids by phospholipases (2). Since cellular AA is almost exclusively sequestered in phospholipids, its availability is important for the synthesis of PG. Free AA is made available to the cell by the action of phospholipase A₂ (PLA₂). There are five different groups of PLA₂. One group of PLA₂, the 85–110 kDa form (also known as Group IV), found in the cytosol, requires only submicromolar levels of calcium for activation (8). It was sequenced and cloned by Clark *et al.* (9) and named the cytosolic PLA₂. Cellular incorporation of ω -6 FAs can be achieved through uptake by LDLr as seen in mesenchymal and epithelial cell types (10–14) or by albumin conjugation of AA as seen in T lymphocytes (15–17).

There is a correlation between the level of AA metabolites and carcinogenesis. Many NSAIDs such as aspirin, indomethacin and sulindac, which inhibit PGE₂ synthesis, also inhibit the growth of colon tumors induced by chemical carcinogens in rodents (18,19). In addition, recent epidemiological studies with large numbers of human patients show that frequent usage of NSAIDs act as a protective agent against colorectal cancers (20–23) and prostate cancer (4,24–30). Our present studies investigate the effects of exogenous AA on molecular and cellular events in the human prostatic adenocarcinoma PC-3 cell line. In this study, we found that albumin-bound AA endocytosed into the cell can induce expression of immediate-early genes *c-fos*, *cox-2* and *cPLA₂*, suggesting that increased availability of AA may play a major role in regulating prostate cancer cell growth.

Materials and methods

Materials

AA and flurbiprofen were purchased from Sigma Chemical (St Louis, MO) or Cayman Chemical (Ann Arbor, MI). Fetal bovine serum (FBS) was purchased from Gibco BRL (Gaithersburg, MD). Antibiotic–antimycotic solution (containing penicillin, streptomycin and amphotericin B) was purchased from Sigma Cell Culture. Fetal calf serum (FCS) was purchased from Hyclone Laboratories (Logan, UT). RPMI medium, L-glutamine and phosphate-buffered saline (PBS) were purchased from Fisher Scientific (Pittsburgh, PA). TRI Reagent™, HEPES buffer and albumin were purchased from Sigma Chemical. *Cox-2* polyclonal antibodies, PGE₂-monoclonal enzyme immunoassay (EIA) kits were purchased from Cayman Chemical. cPLA₂ rabbit polyclonal antibody was purchased from Cell Signaling Technologies (Beverly, MA). β -Actin goat polyclonal antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Peroxidase-conjugated goat anti-rabbit and donkey anti-goat IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Alexa Fluor® 488 conjugated goat anti-rabbit

polyclonal antibody was purchased from Molecular Probes (Eugene, OR). DC protein assay kit and broad range molecular weight markers were purchased from Bio-Rad (Hercules, CA). Bis-Tris gels of 4–12% concentration were purchased from Invitrogen (Carlsbad, CA). Hybond-C extra nitrocellulose membranes were purchased from Amersham Pharmacia (Piscataway, NJ). Supersignal West Pico CL-HRP substrate system was purchased from Pierce-Endrogen (Rockford, IL).

Cell Culture

PC-3 human prostate cancer cell line was obtained from the University of California San Francisco (UCSF) Cell Culture Facility (San Francisco, CA). PC-3 cells were maintained in complete RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 25 mM glucose and 1 mM pyruvate in a 37°C incubator with 5% CO₂ and media was replaced three times a week. Cells were plated in 5% sera for 24 h, switched to 0.3–1% FBS-containing medium in culture dishes for a 48-h downregulation before the cells were treated and harvested for RNA, protein or cell proliferation assay.

RNA isolation

RNA was isolated from cells using Tri-Reagent™ as recommended by the manufacturer and was quantified in the GeneQuant spectrophotometer (Amersham Biotech Pharmacia).

mRNA analysis

Linear semi-quantitative RT-PCR was carried out for analysis of gene expression. The RT-PCR linear range was determined using increasing cycle numbers; the cycle number used for *COX-2* was 34 cycles, for *18S*, 22 cycles and for *cPLA₂*, 42 cycles to measure the response to AA. Areas and intensities of bands were determined using the SigmaGel software (Sigma), and gene expression was represented as the amount of RT-PCR product normalized to *18S* product. Most of the specific PCR primers were designed in this laboratory by MHF and synthesized by Operon Technologies (Alameda, CA). The primers used for amplification were previously described (6), with the exception of *cPLA₂* gene: sense, 5'-GGA TTC TCT GGT GTG ATG AAG G and for anti-sense, 5'-CCC AAT CTG CAA ACA TCA GC. *18S* gene from the same RT was used as internal standard.

Immunofluorescence with Nile red

Cells are treated with 5 µg/ml of AA for 2 h and rinsed in PBS prior to addition of the vital dye, 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile red) to culture for 30 min. Cells were rinsed three times in PBS prior to microscopy and were imaged using a Zeiss axioscop fluorescent microscope (Carl Zeiss, Germany) with an Orca-ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ).

Cell number and measurement of DNA content

Cell counting was performed using the ZBI Coulter Counter (Coulter Electronics, Hialeah, FL), and direct measurement of DNA content was performed (Labsystems; Needham Heights, MA). For DNA or cell number measurement, cells were plated and Hoechst dye #33258 (Calbiochem; San Diego,

CA) or a CyQuant kit (Molecular Probes, Eugene, OR) was used as a DNA fluorescent dye. The fluorescence absorbance was read using the Fluoroskan II spectrofluorometer (Labsystems).

PGE₂ analysis

The PGE₂ levels in media were quantitated using a PGE₂-monoclonal EIA kit according to the manufacturer's protocol. Samples were read at 410 nm with Dynatech MR5000 Microplate Reader (Dynatech Laboratories; Chantilly, VA) and data were analyzed with the BioLinX 2.0 Software (Dynatech Laboratories).

Western blotting

Protein concentration was measured with a DC protein assay kit and western blot analysis was performed as previously described (31).

Immunoprecipitation (IP)

After activation, ice-cold IP cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄ and 1 µg/ml leupeptin was microcentrifuged for 10 min at 4°C at high speed. Supernatant (cell lysate) was collected, and stored at -80°C for subsequent processing. Protein concentration of the cell lysates was determined using DC protein assay kit (Bio-Rad). cPLA₂ rabbit polyclonal antibody was added to 150 µg of cell sample and incubated overnight at 4°C. 20 µl of a 50% agarose bead slurry was added to each sample and incubated for 3 h at 4°C. Cells were sonicated and the cell lysates were microcentrifuged for 30 s at 4°C. Pellets were washed and then resuspended with 20 µl of 3× SDS sample buffer, heated to 100°C for 5 min and then loaded into NuPage 4–12% Bis-Tris minigels and analyzed by western blot against cPLA₂ rabbit polyclonal antibodies as described, and 1 mM PMSF was applied to the cells and incubated on ice for 5 min.

Results

Visualization of uptake of albumin-bound AA

Nile red is a hydrophobic fluorescent stain that allows visualization of lipids at 450–500 and 515–560 nm. At 450–500 nm, a yellow-gold fluorescence shows neutral lipid structures including albumin-coupled AA. At 515–560 nm, a red-orange fluorescence associates with phospholipids, other lipids and hydrophobic protein (32–35). Figure 1A shows lipid content in PC-3 cells that were downregulated prior to the addition of AA. The red-orange fluorescence of the phospholipid is predominant in the downregulated cells. Figure 1B shows PC-3 cells 2½ h after the addition of AA. The yellow-gold fluorescence indicates uptake of AA into the cell most likely facilitated by albumin (17,35).

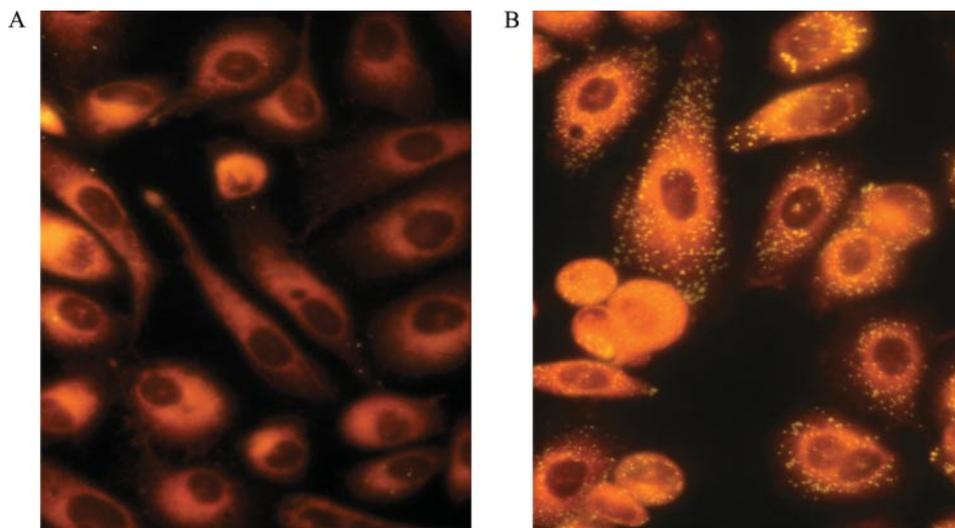


Fig. 1. Uptake of AA by PC-3 cells. PC-3 cells were downregulated and then treated with AA for 2 h before adding Nile red for 30 min. (A) PC-3 cells without AA and (B) PC-3 cells with AA.

The effect of increasing concentrations of exogenous AA on c-fos, cox-2 and cPLA₂ mRNA levels

As shown in Figure 2, AA induces expression of *cox-2*, *cPLA₂* and *c-fos* message in a dose-dependent manner. Measurement of the COX pathway product, PGE₂, also showed a significant increase in a dose-dependent manner that was saturated at 5 µg/ml of AA.

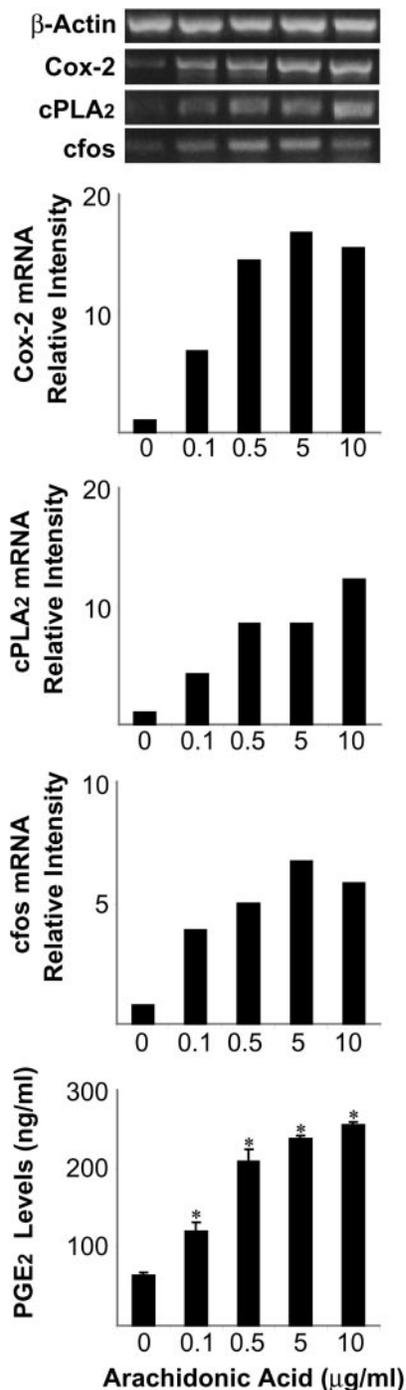


Fig. 2. The effect of increasing concentration of exogenous AA on *cox-2*, *cPLA₂* and *c-fos* mRNA levels. PC-3 cells were serum depleted, then treated with 0, 0.1, 0.5, 5 or 10 µg/ml AA for 3 h and the RNA was isolated. Medium was collected at all concentrations and screened against a monoclonal PGE₂ EIA kit. Each bar represents mean ± SD of PGE₂ levels ($n = 3$, * $P < 0.001$ as compared with AA treatment).

Synthesis of COX-2 and cPLA₂ protein is increased in presence of AA

We analyzed the total COX-2 and cPLA₂ protein present in the cells 2 h after addition of 5 µg/ml AA. In Figure 3, we found that COX-2 and cPLA₂ proteins increased 5-fold and 3-fold, respectively.

The effect of increasing doses of AA with or without flurbiprofen on the endogenous PGE₂ levels

As Figure 2 shows, a correlation existed between increasing *cox-2* and *cPLA₂* mRNA and the increasing endogenous PGE₂ levels in the presence of increasing AA. We also investigated PGE₂ levels in the presence of the flurbiprofen (Figure 4). Although by itself flurbiprofen did not affect endogenous PGE₂ levels, when it is administered with exogenous AA, flurbiprofen decreased the PGE₂ levels by ~1.7-fold.

A flurbiprofen experiment was carried out in the presence of AA to determine whether newly synthesized PGE₂ contributes to *cox-2* mRNA accumulation. In Figure 4, cells treated with 5 µM flurbiprofen reduced *cox-2* mRNA levels by 8-fold and *cPLA₂* mRNA levels by 6-fold compared with AA alone. The fact that flurbiprofen was able to decrease both the endogenous PGE₂ levels as well as the *cox-2* and *cPLA₂* mRNA levels (Figure 4A) suggests that endogenous PGE₂ may play a role in the AA-mediated upregulation of *cox-2* and *cPLA₂* expression. These data support the hypothesis that newly synthesized, endogenous PGE₂ is necessary for regulating the expression of *cox-2* and *cPLA₂* mRNA levels.

Discussion

One of the hallmarks of cellular stimulation in response to hormone, growth factor or phorbol ester activation is the induction of the immediate, early gene expression. Previous evidence shows that a variety of hormones, cytokines and growth factors induce expression of *cox-2* (3) or *cPLA₂* (36–38).

For many decades, fats have been perceived to be only a nutrient fuel and membrane component. In the recent studies, it has been noted that many nutrients serve as controllers of gene transcription. For instance, glucose can cause hormone secretion and polyunsaturated fatty acids have been demonstrated to regulate fatty acid synthases, Spot14 and ApoA1 (39–44). This may be clinically important considering western human dietary intake of fatty acids and high rates of mortality in USA compared with other cultures like Japan. However, latent prostate cancer found at autopsy occurs at the same frequency in Japanese men as in Caucasian males (45). These accumulating data suggest that the higher intake of dietary fat in western society may be a factor in the high rate of prostate cancer incidence in USA (45,46) with African Americans having twice the rate of the Caucasian male. Over the past 60 years in USA, the ratio of dietary intake of ω-6 FA versus ω-3 FA has increased from 2:1 to 25:1 (47); during this same time frame, the incidence of prostate cancer has risen. Even in Japan over the last 30 years, the intake of ω-6 to ω-3 FA has risen to 4:1 from 2:1 (48). Experimentally, there is increasing support to show that FAs stimulate cell growth; however, the mechanism is not fully understood.

Our data show that the essential fatty acid AA causes induction of the immediate early genes *c-fos* and *cox-2*, which is co-regulated with the induction of *cPLA₂* message. There is also a dose-dependent synthesis of PGE₂ (Figure 2). When COX-2 is inhibited by NSAIDs, dose-dependent production

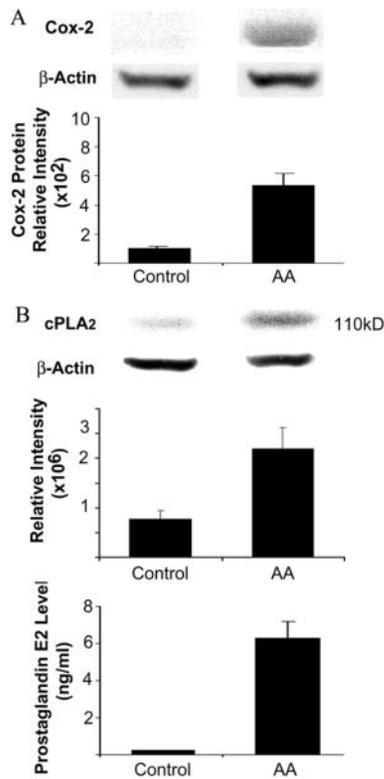


Fig. 3. COX-2 and cPLA₂ protein and product is increased in presence of AA. Cells were grown and downregulated, then 5 μg/ml of AA was added for a 2-h incubation period. Media were collected and stored at -80°C until analysis for PGE₂. Proteins were screened with (A) COX-2 antibody with western blot, (B) cPLA₂ content of each sample was immunoprecipitated and then analyzed with western blot. Relative intensities were normalized to the β-actin. Each bar represents mean ± SD intensity of three independent samples ($n = 3$).

of PGE₂, gene induction and cell growth are blocked, suggesting a role for PGE₂ in signal transduction of the essential fatty acids. Blocking cPLA₂ also reduced cell number (Figure 4).

The data presented in the present paper show, for the first time, that free fatty acids (albumin-bound AA) coordinately upregulate the expression of *cox-2* and *cPLA₂* mRNA via the formation of PGE₂ in a human prostate cancer cell line. We have shown previously that dmPGE₂, a stable analog of PGE₂, increases cell growth and upregulates both protein and mRNA expression of COX-2 (4,5). This upregulation depends upon new synthesis of PGE₂, since treatment with an NSAID, such as flurbiprofen, reverses the PGE₂-induced increase in *cox-2* transcript levels. Herschman *et al.* (3) have proposed a model in which there are two different pathways by which PGs may be synthesized. These pathways use distinct pools of AA and consist of (i) an intracellular pathway by which AA released from membrane phospholipid, following ligand stimulation, is made available only to COX-2 and (ii) a transcellular pathway by which soluble PLA₂ (sPLA₂) mobilizes AA from a different pool of membrane phospholipids than those used in (i) to be available to COX-1. Here we demonstrate that extracellular fatty acid bound to albumin enters the cell within minutes and causes an induction of message, proteins of COX-2 and cPLA₂, and growth.

Exogenous AA increases cellular growth and total DNA content, which is reversible by a COX inhibitor such as flurbiprofen. The data also suggest that increased levels of PGE₂ regulate gene expression and stimulate cellular growth. This

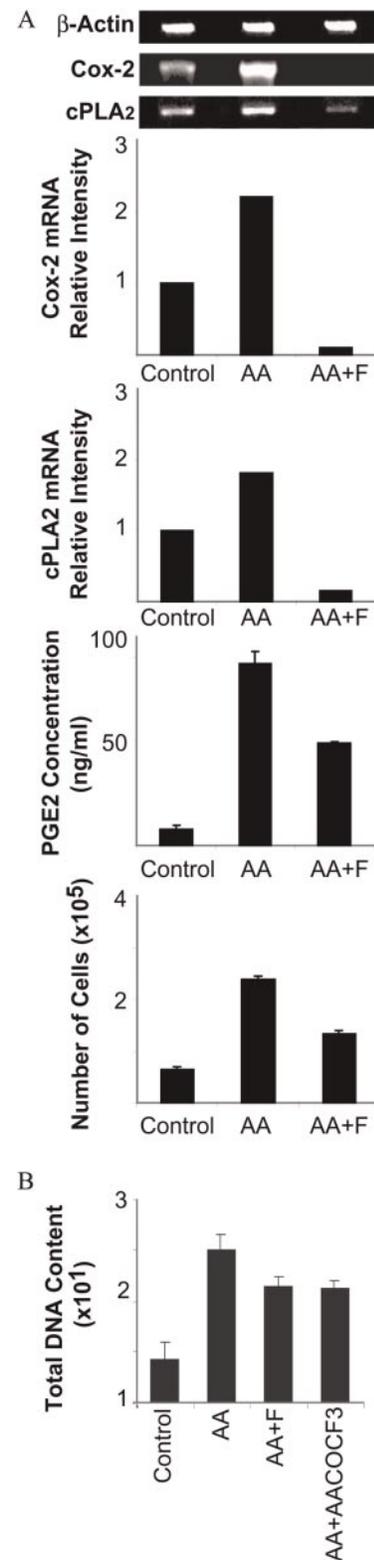


Fig. 4. Flurbiprofen inhibits *cox-2* and *cPLA₂* upregulation, hence inhibits AA induction of PGE₂ in PC-3 prostate cancer cells. PC-3 prostate cancer cells were serum-deprived and then treated with indicated concentration of flurbiprofen 1 h prior to a 3-h treatment of 5 μg/ml of AA combined with 1.25 mg/ml albumin. (A) 20 μg of protein was loaded and screened against COX-2. Representative western blot bands are shown in each graph; each data point is the mean ± SD of independent triplicate blots for each treatment ($n = 3$, * $P < 0.001$ as compared with AA treatment). The membrane was reprobbed against β-Actin. (B) Medium was collected and PGE₂ levels were determined. Each bar represents mean ± SD for each treatment ($n = 3$, * $P < 0.001$ as compared with AA treatment).

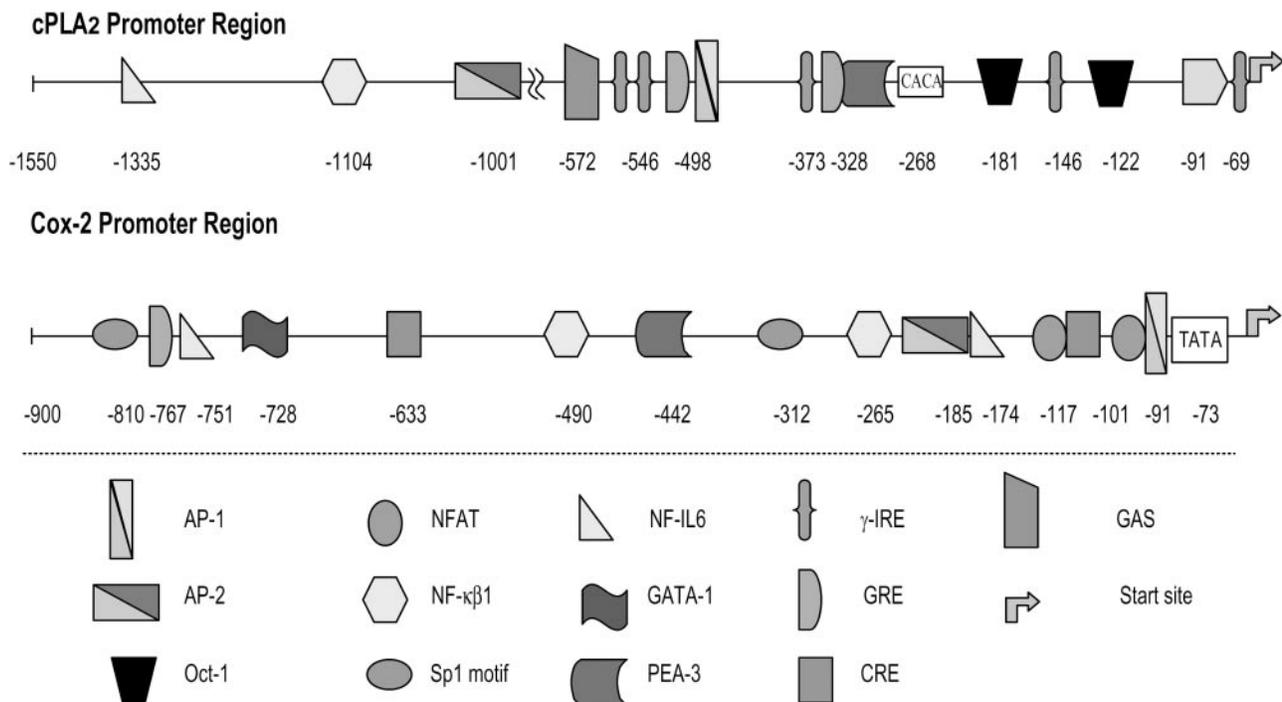


Fig. 5. Illustration of *cPLA₂* and *Cox-2* promoter region. The promoter region sequence of *cPLA₂* and *cox-2* were loaded onto DNAsis MAX software, possible motifs in the promoter regions are identified and confirmed with literature. Confirmed DNA motifs are illustrated. See online Supplementary material for a colour version of this Figure.

finding is consistent with a recent report shown in NIH/3T3 cells: COX-1 is localized predominantly in the endoplasmic reticulum, whereas COX-2 is localized in both the endoplasmic reticulum and the nuclear envelope (49,50). In the present study, we also found that the majority of the AA induced newly formed COX-2 to be localized in the endoplasmic reticulum and nuclear envelope in prostate cancer cells (data not shown).

Exogenous AA upregulates *cox-2* and *cPLA₂* transcript levels in a dose-dependent manner (Figure 2). We did not detect the expression of *cox-1* mRNA in PC-3 cells under any culture condition. Similarly, in previous work we have shown that expression of *cox-1* mRNA was not detected in colorectal carcinoma DiFi cells, (5), suggesting that PGE₂ is synthesized mainly by the action of COX-2 in these two cell lines. Herschman *et al.* (3) suggested the presence of two different PG synthesis pathways, both of which depend upon the pool of free cellular arachidonate and the COX (51,52). In PC-3 cells, however, it is apparent that albumin-bound exogenous AA becomes a direct substrate for *cox-2*, leading to synthesis of PGE₂. PGE₂ then upregulates growth-related gene expression, such as *c-fos*, *cox-2* and *cPLA₂*, resulting in increased enzyme synthesis, cell growth and proliferation.

cPLA₂ mRNA expression is activated by exogenous AA (Figures 2–4). This upregulation correlates with new synthesis of PGE₂ (Figures 2 and 4). To our knowledge, this is the first evidence to suggest that *cPLA₂* expression is upregulated by AA and its metabolite, PGE₂. *cPLA₂* mRNA and/or protein has been reported to be inducible following administration of various growth factors or cytokines, including tumor necrosis factor (53), transforming growth factor-β and interleukin-1β (54), thrombin (37,55) and c-kit ligand (56,57). It has been proposed that *cPLA₂* is activated in response to agents that increase intracellular Ca²⁺, which facilitates translocation of *cPLA₂* from the cytosol to the cell membrane, where its

substrate is localized (58). AA has been implicated as an ionophore (59) and its product PGE₂ is a known ionophore (60). Since treatment of cells with flurbiprofen reverses the increase in expression caused by endogenous AA (Figure 4), it follows that the increased expression of these genes and enzymes depend upon endogenous synthesis of PGE₂. In PC-3 cells, the fact that endogenous PGE₂ levels also increased dose-dependently following AA treatment, suggests that the upregulation of *cPLA₂* mRNA levels may be as a result of the new PGE₂ synthesis, rather than a direct effect of AA. The activation of *cPLA₂* expression may involve the same mechanism as that of *cox-2*, since the two genes have several identical promoter elements. Although the *cPLA₂* gene contains no traditional TATA box, its 5'-flanking region, the promoter, does include responsive elements to transcription factors: NF-κB, NF-IL6, AP-1, AP-2 and PEA-3 (61–63) (Figure 5) that are in common with the *cox-2* gene. Moreover, the human and rat *cPLA₂* genes have been localized to the same region of chromosome I near COX-2, suggesting the possibility of coordinate regulation between the two genes (64–66). We are currently investigating the cellular and molecular mechanisms by which PGE₂ induces *cPLA₂* and *cox-2* mRNA accumulation.

This study suggests that AA increases PC-3 prostate tumor cell growth, total DNA content and endogenous PGE₂ levels via induction of *c-fos*, *cPLA₂* and *cox-2* mRNA transcription. These findings are of interest since this cell line has previously been shown to be responsive to growth stimulation by the ω-6 polyunsaturated fatty acid, LA (an essential fatty acid precursor of AA). This growth stimulation is thought to be dependent upon eicosanoid biosynthesis (67), and our data support this finding by providing evidence that AA, LA or PGE₂ serve as a non-peptide growth factor in cancer cells (4,5,68). Cellular levels of AA are regulated primarily by the concentrations of AA that the cell is normally exposed to

in vivo. A cell can acquire AA through several means, including (i) from serum low density lipoprotein, which sequesters unsaturated fatty acids, primarily arachidonic acid, and is taken up by the cell through an LDL receptor-mediated mechanism (14) and (ii) from an AA-albumin complex that is distributed freely in serum (69,70). We provide further evidence that AA regulates gene expression, and protein synthesis and proliferation, which are blocked by NSAIDs, pointing to regulation by PGE₂. Although several reports have shown that COX-2 specific inhibitors are effective in reducing prostate cancer growth (71,72), recent findings have revealed that some of these specific COX-2 inhibitors have unwanted side effects. It is possible that cPLA₂ may be an alternate upstream target for pharmaceutical intervention of prostate cancer.

In addition, cellular AA level seems to be important in the maintenance of growth and homeostasis of prostate cancer cells *in vitro* and it is possible that the essential fatty acids also play a role clinically in individuals eating a Western diet which has increased 25-fold in ω-6 FA content during the last century (47). The data in this paper support the hypothesis that exogenous AA and newly synthesized PGE₂ play a physiological role in cancer cell growth and gene induction. The data bring us one step closer to defining a molecular link between dietary fatty acid intake and increased cancer growth.

Supplementary material

Supplementary material is available at <http://carcin.oxfordjournals.org/>

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