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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. 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 (1–5). 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 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® (Sigma Chemical). Cox-2 monoclonal antibodies, PGE2 monoclonal enzyme immunoasay (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 Bio-technologies (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

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.
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 incubated with 5% CO2 and media was replaced three times a week.

RNA was isolated from cells using Tri-Reagent (Amersham Biotech Pharmacia). The RT–PCR linear range was determined using increasing cycle numbers; the cycle number used for each reaction was 34 cycles, for COX-2, 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 rRNA. 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′-GGG TTC TCT GGT GTG ATG AAG G and for anti-sense, 5′-CCC AAT CTG CAA ACA TCA GC. 18S gene from the same sample was used as an internal standard.

Immunofluorescence with nile red
PC-3 cells were downregulated and then treated with AA for 2 h before adding nile red for 30 min. A yellow-gold fluorescence shows neutral lipid structures (17,35). Nile red is a hydrophobic fluorescent stain that allows visualization of lipids at 450–500 nm and 515–560 nm. At 450–500 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 21/2 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.

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.

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...
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. 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).

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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 reprobed 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).
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 \(\text{cox-2}\) and \(\text{cPLA2}\) transcript levels in a dose-dependent manner (Figure 2). We did not detect the expression of \(\text{cox-1}\) mRNA in PC-3 cells under any culture condition. Similarly, in previous work we have shown that expression of \(\text{cox-1}\) mRNA was not detected in colorectal carcinoma DiFi cells, (5), suggesting that PGE 2 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 \(\text{cox-2}\), leading to synthesis of PGE\(_2\), PGE\(_2\) then upregulates growth-related gene expression, such as \(\text{c-fos, cox-2}\) and \(\text{cPLA2}\), resulting in increased enzyme synthesis, cell growth and proliferation. \(\text{cPLA2}\) mRNA expression is activated by exogenous AA (Figures 2–4). This upregulation correlates with new synthesis of PGE\(_2\) (Figures 2 and 4). To our knowledge, this is the first evidence to suggest that \(\text{cPLA2}\) expression is upregulated by AA and its metabolite, PGE\(_2\), \(\text{cPLA2}\) 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-\(\beta\) and interleukin-1\(\beta\) (54), thrombin (57,55) and e-kit ligand (56,57). It has been proposed that \(\text{cPLA2}\) is activated in response to agents that increase intracellular Ca\(^{2+}\), which facilitates translocation of \(\text{cPLA2}\) from the cytosol to the cell membrane, where its substrate is localized (58). AA has been implicated as an ionophore (59) and its product PGE\(_2\) is a known ionophore (60). Since treatment of cells with flurbiprofen reverses the increase in expression caused by exogenous AA (Figure 4), it follows that the increased expression of these genes and enzymes depend upon endogenous synthesis of PGE\(_2\). In PC-3 cells, the fact that endogenous PGE\(_2\) levels also increased dose-dependently following AA treatment, suggests that the upregulation of \(\text{cPLA2}\) mRNA levels may be as a result of the new PGE\(_2\) synthesis, rather than a direct effect of AA. The activation of \(\text{cPLA2}\) expression may involve the same mechanism as that of \(\text{cox-2}\), since the two genes have several identical promoter elements. Although the \(\text{cPLA2}\) gene contains no traditional TATA box, its 5'-flanking region, the promoter, does include responsive elements to transcription factors: NF-\(\kappa\)B, NF-IL6, AP-1, AP-2 and PEA-3 (61–63) (Figure 5) that are in common with the \(\text{cox-2}\) gene. Moreover, the human and rat \(\text{cPLA2}\) 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 PGE\(_2\) induces \(\text{cPLA2}\) and \(\text{cox-2}\) mRNA accumulation.

This study suggests that AA increases PC-3 prostate tumor cell growth, total DNA content and endogenous PGE\(_2\) levels via induction of \(\text{c-fos, cPLA2}\) and \(\text{cox-2}\) transcript. These findings are of interest since this cell line has previously been shown to be responsive to growth stimulation by the \(\omega-6\) 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 PGE\(_2\) serve as a non-polypeptide 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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**References**


