Prostaglandin $E_2$ and the protein kinase A pathway mediate arachidonic acid induction of $c$-fos in human prostate cancer cells

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Summary Arachidonic acid (AA) is the precursor for prostaglandin $E_2$ ($PGE_2$) synthesis and increases growth of prostate cancer cells. To further elucidate the mechanisms involved in AA-induced prostate cell growth, induction of $c$-fos expression by AA was investigated in a human prostate cancer cell line, PC-3. $c$-fos mRNA was induced shortly after addition of AA, along with a remarkable increase in $PGE_2$ production. $c$-fos expression and $PGE_2$ production induced by AA was blocked by a cyclo-oxygenase inhibitor, flurbiprofen, suggesting that $PGE_2$ mediates $c$-fos induction. Protein kinase A (PKA) inhibitor H-89 abolished induction of $c$-fos expression by AA, and partially inhibited $PGE_2$ production. Protein kinase C (PKC) inhibitor GF109203X had no significant effect on $c$-fos expression or $PGE_2$ production. Expression of prostaglandin (EP) receptors, which mediate signal transduction from $PGE_2$ to the cells, was examined by reverse transcription polymerase chain reaction in several human prostate cell lines. EP4 and EP2, which are coupled to the PKA signalling pathway, were expressed in all cells tested. Expression of EP1, which activates the PKC pathway, was not detected. The current study showed that induction of the immediate early gene $c$-fos by AA is mediated by $PGE_2$, which activates the PKA pathway via the EP2/4 receptor in the PC-3 cells. © 2000 Cancer Research Campaign

Keywords: prostate cancer; $c$-fos; arachidonic acid; prostaglandin $E_2$; protein kinase; gene expression; prostaglandin receptor; EP4

Dietary fatty acid intake is associated with the risk of development and progression of prostate, colon and breast cancer (Marnett, 1992; Rose, 1997). In vitro and in vivo studies suggest that the availability of polyunsaturated fatty acids contributes to increased cancer cell growth, and that inhibitors of the eicosanoids synthesis pathway inhibit cell proliferation (Rose and Connolly, 1991; Connolly et al, 1997).

Arachidonic acid (AA) is derived from the essential polyunsaturated fatty acid, linoleic acid, which is commonly available in dietary fat. It is the major precursor of biologically active eicosanoids, which include prostaglandins, thromboxanes and leukotrienes. Among these is prostaglandin $E_2$ ($PGE_2$), a product catalysed by the key enzyme cyclo-oxygenase (COX, EC 1.14.99.1). Two isosforms of COX exist in human prostate cells. COX-1 is a constitutively expressed enzyme, whereas COX-2 is inducible. O’Neill and Ford-Hutchinson (1993) have reported that the human prostate cells express similar amounts of COX-1 and COX-2. In human prostate tissues, $PGE_2$ is the only significant eicosanoid produced (Chaudry et al, 1994). $PGE_2$ induces a variety of cell responses depending on the tissue type and the receptors involved, such as immune regulation (Monick et al, 1987; Juzan et al, 1992; Marnett, 1992), smooth muscle contraction (Coleman and Kennedy, 1985) and regulation of water re-adsorption in kidney (Smith, 1989). Previous studies with osteoblasts and prostate cancer cells have demonstrated that $PGE_2$ stimulates cell growth (Raisz et al, 1993; Tjandrawinata et al, 1997).

Studies from this laboratory indicate that AA stimulates growth of a prostate cell line, PC-3 (manuscript submitted). Because of the role of $PGE_2$ in prostate cancer cell growth, we hypothesized that AA had the stimulatory effect on the prostate cancer cells through the activity of its metabolite $PGE_2$, and aimed to understand the signal transduction events following AA administration which lead to the growth of the cells. After being synthesized and exported out of the cells, $PGE_2$ exerts its functions by interacting with the $PGE_2$ receptors (EPs), which are 7-domain transmembrane cell surface receptors. Four subtypes of EP receptors have been identified and characterized. These receptors are coupled to G proteins, and activate or inhibit second messenger systems inside the cell. EP1 causes influx of Ca$^{2+}$ and activation of protein kinase C (PKC); receptors EP2 and EP4 activate the adenylate cyclase which increases cellular cyclic AMP level and activates protein kinase A (PKA); EP3 signals primarily through an inhibitory G protein to decrease intracellular cyclic AMP levels (Nagishi et al, 1995).

Despite the important association of AA and $PGE_2$ with prostate cancer, the signals mediating the biological functions of these molecules in prostate cancer cells are not fully understood. The signalling pathways mediating AA- or $PGE_2$-induced cell growth or expression of growth-related proto-oncogenes have been investigated in a number of studies with varying conclusions. In bone cells, a PKA-mediated mechanism has been suggested (Fitzgerald et al, 1999; Weinreb et al, 1999). A study of smooth muscle cells demonstrated a role for PKC as a mediator of AA-induced $c$-fos
expression (Rao et al, 1993). In the Swiss 3T3 fibroblast cells, however, there are conflicting data on whether PKC or PKA is involved (Kacich et al, 1988; Mehmet et al, 1990; Danesch et al, 1994). Our results indicate that the PKA-dependent pathway via the EP4 receptor is responsible for mediating AA-induced c-fos expression in human prostate cancer cells.

MATERIALS AND METHODS

Materials

AA, flurbiprofen, and 12-O-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma (St Louis, MO, USA). PKC inhibitor GF109203X and PKA inhibitor H-89 were from BIOMOL (Plymouth Meeting, PA, USA). PGE2 was from Oxford (Oxford, MI, USA). RPMI-1640 medium, L-glutamine, and antibiotics were from UCSF Cell Facility (San Francisco, CA, USA). Fetal calf serum (FCS) was purchased from Hyclone Laboratories (Logan, UT, USA).

Cell culture

The PC-3, DU145 and LNCaP human prostate cancer cell lines were cultured in complete RPMI-1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 25 mM glucose, 1 mM pyruvic acid, 100 units ml$^{-1}$ penicillin, 100 mg ml$^{-1}$ streptomycin and 0.25 mg ml$^{-1}$ amphotericin B. The PrEC normal human prostate epithelial cells (Clonetics, San Diego, CA, USA) were maintained in the PrEGM medium (Clonetics). All cells were cultured with 5% carbon dioxide at 37°C in the culture medium samples were determined using a PGE2 monoclonal enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA), following the protocols recommended by the company. An aliquot of 1.5 mg RNA was reverse transcribed in 30 µL of buffer. Five microlitres of the reverse transcription reaction was PCR-amplified using specific primers. A temperature cycle in PCR was 94°C for 1 min 40 s, 63°C for 1 min 10 s and 72°C for 1 min 40 s. PCR was carried out in a Robocycler 40 (Stratagene, San Diego, CA, USA) for various cycle numbers depending on the primers used (the cycle numbers are described in the legends for Tables and Figures). PCR cycle numbers were maintained within a linear amplification range. The PCR products were separated by electrophoresis in 2% agarose gels. The gels were photographed and DNA bands of interest were scanned at 400 dpi with an Epson Perfection 636 scanner. Areas and densities of the DNA bands were determined using the SigmaGel software (Sigma).

PGE2 assay

An aliquot of culture medium was collected and frozen at −70°C before the cells were harvested for RNA isolation. PGE2 levels in the culture medium samples were determined using a PGE2, monoclonal enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA), following the protocols recommended by the company. A Dynatech MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA, USA) was used to read the assay results. Data were analysed using the BioLinx 2.0 software (Dynatech).

Statistical analysis

For each treatment in the experiments, three independent samples were seeded, treated and their RNA or media samples analysed separately. Mean and standard deviation (s.d.) of the three samples

| Table 1  | Primers used in RT-PCR |
|---------|------------------------|
| Gene    | Orientation  | Sequence                           | Product size (bp) |
| c-fos   | Sense       | 5' GAATAGATGCTGCAGCCAAATGCCAGCA  | 236              |
|         | Anti-sense  | 5' CAATCAACCGAAAGCTCAAGAAGAAGCA  |                  |
| Cyclophilin | Sense     | 5' CTCCTCCTTTGAGCGTTTGGCA       | 628              |
|         | Anti-sense  | 5' ATATCTACACACTTTTCAGGCGAAT   |                  |
| EP1     | Sense       | 5' CCACACCTCTGTCGTTGTCG         | 1037             |
|         | Anti-sense  | 5' GGTGGCTGCTGTCGTTG          |                  |
| EP2     | Sense       | 5' CCACTTGCGCCACCTGTTG         | 784              |
|         | Anti-sense  | 5' GGTGGCTGCTGTCGTTG         |                  |
| EP3     | Sense       | 5' GCCATCAACCTCTCAGGAGA        | 837              |
|         | Anti-sense  | 5' GAGAGCGAGAACGACG            |                  |
| EP4     | Sense       | 5' AGATGTCCTGTCGCTGTTG         | 344              |
|         | Anti-sense  | 5' GAGATGTCCTGTCGCTGTTG        |                  |
are shown in the figures. One-way analysis of variance (ANOVA) was performed using the SigmaStat (Sigma) software to obtain the P-values for comparison between treatments.

RESULTS

AA transiently increases c-fos mRNA expression in PC-3 cells

A previous study from this laboratory demonstrated that AA induces c-fos expression in the PC-3 human prostate cancer cells (manuscript submitted). In this study, AA was added to the PC-3 cells at a concentration of 1 µg ml⁻¹ since previous work demonstrated that c-fos expression can be up-regulated with as low as 0.1 µg ml⁻¹ of AA, and that the effect is near maximum when the AA concentration is at 0.5 µg ml⁻¹. Fifteen, 30, 60 and 180 min after addition of AA, total RNA was isolated from the cells and was subjected to RT-PCR analysis to observe level of c-fos mRNA. Table 2 summarizes the relative level of c-fos product after being normalized to the internal control, cyclophilin. c-fos message was induced as early as 15 min after AA addition to cell culture, and was greater than 1.9-fold higher than the non-treated control at 1 h. Three hours after AA addition, c-fos mRNA level began to decrease, suggesting that c-fos induction was transient and was regulated by other downstream events. Based on these observations, cells and medium samples were collected 1 h after AA was added to culture media for all subsequent AA-induction experiments.

AA-induced c-fos expression is mediated by PGE₂

Because AA is the precursor of PGE₂, it was speculated that the AA-induced c-fos expression was due to increased PGE₂ production by the PC-3 cells. Culture medium samples collected from the experiment presented in Table 2 were analysed for their PGE₂ levels. As expected, PGE₂ production of the PC-3 cells increased shortly after its precursor, AA, became available to the cells and PGE₂ level remained elevated for at least 3 h (Table 2).

In order to confirm that PGE₂ mediated AA-induced c-fos expression, we blocked PGE₂ synthesis with a non-steroidal anti-inflammatory drug (NSAID), Flurbiprofen, which suppresses cyclo-oxygenases and subsequently PGE₂ production, was added to the cells before the AA treatment. As shown in Figure 1A, c-fos message induction by AA was inhibited by flurbiprofen, suggesting an important role for PGE₂ production in this event. Increased c-fos expression in PC-3 cells was similarly achieved by PGE₂ treatment (Figure 1B). The effect of flurbiprofen on PGE₂ production by PC-3 cells was measured. PGE₂ level of the culture media was increased more than sevenfold by AA, but this increase was abolished by flurbiprofen treatment (Figure 2). These data strongly suggest that AA increases c-fos expression via a PGE₂ mechanism.

AA-induced c-fos expression is dependent on the PKA signalling pathway

The EP receptors for PGE₂ are coupled to either PKC or PKA pathways. To determine which EP receptor signalling pathway is

| Incubation time with AA | Relative c-fos product | PGE₂ concentration (µg ml⁻¹) |
|-------------------------|------------------------|-----------------------------|
| Control                 | 2.81 ± 0.26            | 80 ± 34                     |
| 15 min                  | 3.59 ± 1.09            | 361 ± 107                   |
| 30 min                  | 4.42 ± 0.47            | 402 ± 18                    |
| 1 h                     | 5.46 ± 0.83            | 425 ± 52                    |
| 3 h                     | 3.62 ± 0.69            | 558 ± 131                   |

Total RNA was isolated from the PC-3 cells after incubation with AA (1 µg ml⁻¹) for a time period indicated in the Table. RT-PCR with c-fos and cyclophilin primers was performed on the RNA samples. PCR reactions with c-fos and cyclophilin primers had 29 and 19 temperature cycles respectively. The amount of c-fos RT-PCR product was normalized to that of the cyclophilin product. Culture medium samples from the same experiment were assayed for PGE₂ level. The numbers represent mean ± s.d. of three independent samples.
involved in the AA-induced c-fos expression in the PC-3 cells, specific protein kinase inhibitors were used to treat the cells before AA was added. Treatment with H-89, a selective inhibitor of PKA (Chijiwa et al, 1990), resulted in loss of c-fos induction by AA (Figure 3). Inhibition of PKA also lowered PGE2 production by 2.8-fold after AA was added to the cells (Figure 4). This reduced
PGE₂ level, however, was still 4.6-fold higher than the control samples, or 3.7-fold higher than the samples treated with H-89 but not with AA.

The specific PKC inhibitor, GF109203X (Toullec et al, 1991), was added to the cells prior to AA treatment in order to determine whether PKC is involved in c-fos induction in PC-3 cells. Figure 5A shows that inhibition of PKC did not significantly block AA-induced c-fos expression. The inhibitory effect of GF109203X was demonstrated by its strong inhibition of c-fos expression induced by TPA, which is a potent activator of PKC (Figure 5B). AA-induced PGE₂ production in PC-3 cells was also not affected by treatment with GF109203X (Figure 6).

Taken together, the results suggest that the PKA but not PKC signalling pathway is responsible for PGE₂ signal transduction that leads to increased c-fos expression after addition of AA. Therefore, the EP1 receptor, which activates the PKC pathway, is not likely to be involved in receiving the PGE₂ signal in PC-3 cells.

**Human prostate cells express the prostaglandin receptors EP4 and EP2**

Expression of different EP receptors was examined in three human prostate cancer cell lines (PC-3, LNCaP, and DU145) and in PrEC normal prostate cells. Cells were grown in their normal growth media before harvested for RNA isolation and RT-PCR. A strong band was detected when the RT samples were PCR-amplified with EP4 primers after 31 cycles. This band was present in all four cell lines examined. Using the EP2 primers, only a weak band was present in all the cells after 40 cycles of amplification, suggesting a very low copy number. No RT-PCR product of the expected size was detected in any of the cell lines when EP1 or EP3 primers were used (Figure 7), even after varying the magnesium concentrations in PCR (data not shown). We conclude that the human prostate cells express EP4 and EP2 receptors and probably not EP1 or EP3.

**DISCUSSION**

The current study suggests that the mechanism by which AA increases c-fos expression and growth in PC-3 cells involves production of PGE₂ from AA, binding of PGE₂ to the EP4 and/or EP2 receptors, and subsequent activation of the PKA pathway which eventually leads to expression of growth-related genes (Figure 8). Under normal physiological conditions, AA is released from membrane phospholipids by phospholipases upon stimulation to synthesize eicosanoids (Smith, 1989). Because human cells can not synthesize AA de novo, AA has to be obtained directly from the diet, or synthesized from linoleic acid, which is also an essential fatty acid. AA is then present in serum either as an albumin-bound acid, or as part of lipids present in lipoproteins. Habenicht et al (1990) have demonstrated that cholesterol-γAA is taken up by the cells via the LDL receptor pathway. Serum albumin-bound AA enters the cells by endocytosis (Geuskens et al, 1994; Iturralde et al, 1991; Uriel et al, 1994). After transportation into the cells, AA is incorporated into the lipid pool as part of phospholipids (Chilton et al, 1996). The mechanisms by which prostaglandins (synthesized from AA via the cyclo-oxygenase pathway) facilitate cancer transformation include generation of mutagens, stimulation of cell growth, tumour promotion and immune suppression (Marnett, 1992). The importance of the cyclo-oxygenase products in prostate cancer has also been demonstrated by a recent study (Liu et al, 1998) in which a specific COX-2 inhibitor induced apoptosis of the LNCaP human prostate cell line.

*c-fos* is one of the earliest induced growth response genes (Lau and Nathans, 1987; Angel and Karin, 1991). In this study, expression of *c-fos* measured by semi-quantitative RT-PCR increased > 1.9-fold when prostate cancer cells were treated with AA in all experiments. The increased expression of *c-fos* may, at least in part, account for the increased PC-3 cell growth as previously observed (Tjandrawinata et al, 1997). Our results strongly suggest that AA induces c-fos expression through the biological activity of
its product PGE$_2$ for two reasons. First, PGE$_2$ had a similar effect on c-fos expression in PC-3 cells; and second, treatment with a cyclo-oxygenase inhibitor suppressed the induction of c-fos expression by AA. To understand the signal transduction pathway(s) involved in AA-induced c-fos expression in the prostate cancer cells, specific protein kinase inhibitors were used. The results clearly indicate a role of PKA, but not PKC, in the activation of the signal transduction event. Therefore, the prostaglandin receptor(s) that mediate this event must be EP2 and/or EP4, which are linked to the PKA pathway. Consistent with the results from protein kinase inhibition experiments, RT-PCR detected EP4 as the major EP receptor expressed in human prostate cells, with a small amount of EP2 expression also detected. Expression of EP1 and EP3 was not detected. Expression of specific types of EP receptors appears to be the mechanism used by different cell types for carrying out various physiological functions of the eicosanoids.

Interestingly, the PKA inhibitor H-89 partially inhibited PGE$_2$ production from AA. This, however, was unlikely the cause for the complete loss of AA-induction of c-fos expression, because the PGE level in presence of H-89 and AA was still much higher than in control samples. Two possibilities are proposed to explain this partial inhibition of PGE$_2$ synthesis. First, COX-2 is a feed-forward enzyme up-regulated by its product PGE$_2$ (Pilbeam et al, 1995; Tjandra Ninita et al, 1997). Inhibition of PKA blocks the pathway by which PGE$_2$ exerts its cellular functions through the EP4/2 receptors in PC-3 cells. This presumably prevents further activation of cyclo-oxygenases, thus decreasing the amount of subsequent PGE$_2$ production. Second, it is possible that other stimulating factors are needed for conversion of AA into PGE$_2$, and the receptors of AA induced mediators of AA expression in the human prostate cancer cell line PC-3.

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