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UP-REGULATION OF CYCLOOXYGENASE-2 BY PRODUCT-PROSTAGLANDIN E_2

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ABSTRACT

The development of prostate cancer has been linked to high level of dietary fat intake. Our laboratory investigates the connection between cancer cell growth and fatty acid products. Studying human prostatic carcinoma PC-3 cells, we found that prostaglandin E_2 (PGE_2) increased cell growth and up-regulated the gene expression of its own synthesizing enzyme, cyclooxygenase-2 (COX-2). PGE_2 increased COX-2 mRNA expression dose-dependently with the highest levels of stimulation seen at the 3-hour period following PGE_2 addition. The NSAID flurbiprofen (5 μM), in the presence of exogenous PGE_2, inhibited the up-regulation of COX-2 mRNA and cell growth. These data suggest that the levels of local intracellular PGE_2 play a major role in the growth of prostate cancer cells through an activation of COX-2 gene expression.

INTRODUCTION

Mammalian cells appear to contain at least two isozymes of cyclooxygenase; COX-1 and COX-2. COX-1 is a well characterized, constitutively expressed enzyme, while COX-2 mRNA and/or protein has been shown to be inducible in varieties of cells following addition of various growth-promoting stimuli such as serum (1, 2, 3). COX-1 and COX-2 polypeptides share 61 % primary sequence identity (4).

Recent studies by Rose and Connoly have shown in human prostate cancer cell lines that growth of the androgen-unresponsive PC-3 prostate cancer cells are stimulated and inhibited *in vitro* by the addition of the omega-6 polyunsaturated linoleic acid and NSAIDS such as indomethacin, esculetin and piroxicam, respectively (5, 6). The growth effects of essential fatty acids appear to involve both prostaglandins and leukotrienes and to interconnect with regulation by EGF-related polypeptides (6, 7). Wahle and co-workers have

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also shown that human malignant prostatic tissues had significantly reduced arachidonic acid concentration as compared to benign tissue (8). When these investigators followed the metabolism of labeled arachidonic acid, significant amounts of the radioactive label was found in PGE_2 in both benign and malignant prostatic tissues, with the malignant tissues converting radiolabelled AA to PGE_2 at an almost 10-fold higher rate compared to benign tissues (9). The data suggest a specific role for PGE_2 in maintaining the growth of malignant prostatic tissues.

Our studies were performed to determine the effects of exogenous PGE_2 on the COX-2 expression in the human prostatic adenocarcinoma PC-3 cell line. We have shown previously that prostaglandin E_2 can act as an autocrine growth factor in the growth of osteoblasts 3T3 cells (10). PGE_2 upregulated the expression of early immediate genes such as *c-fos* and *c-jun* as well as increased the DNA synthesis and the cell number of the bone cells in comparison to the control cells (10, 11). We reasoned that if PC-3 is responsive to growth stimulation by linoleic acid, then it may be also responsive to growth stimulation by PGE_2. Indeed, our data suggest that PGE_2 at the micromolar level is able to stimulate PC-3 cell growth partly through up-regulation of COX-2 gene expression.

MATERIALS AND METHODS

Cell Culture

Human Prostatic Carcinoma PC-3 cells were grown in T-150 flasks with 10% fetal bovine serum-containing RPMI-1640 medium (UCSF Cell Culture Facility, San Francisco, CA) supplemented with L-glutamine and antibiotics (Sigma Cell Culture, St. Louis, MO). Cells were maintained at high density in a 37°C incubator with 5% CO_2. Twenty four hours prior to cell platings, cell stocks were fed with fresh 10% FBS-containing medium. Cells were were plated out in 0.3% FBS-containing medium and incubated for another 48 hours to synchronize the growth and to deplete any residual serum growth factors that might be prsent in the culture medium. Each experiment was done at least three times, and the results were found to be consistent.

RNA Isolation

RNA was extracted and was isolated by the acid guanidium thiocyanate/phenol/chloroform extraction method (RNA Stat-60 reagent) according to the procedure recommended by the manufacturer (TelTest "B", Inc.; Friendswood, TX), and was precipitated overnight using isopropanol. The RNA was then dissolved in diethylpyrocarbonate-treated (DEPC) water and was subjected to further quantitation on GeneQuant spectrophotometer (Pharmacia LKB Biotechnology; Piscataway, NJ).

RT-PCR analysis

RNA was reverse-transcribed in the presence of deoxynucleotides (Boehringer Mannheim; Indianapolis, IN), Oligo $(dT)_{12-18}$ primer (Gibco BRL), RNase-Inhibitor (Boehringer Mannheim), M-MLV Reverse Transcriptase (Gibco BRL), first strand and DEPC-treated water. The RT was carried out in Robocycler 40 Temperature Cycler (Stratagene; San Diego, CA). The PCR portion was carried out in tubes containing single stranded cDNA from RT sample, $MgCl_2$ (Gibco BRL), deoxynucleotides (Boehringer Mannheim), *Taq* DNA Polym-

erase (Gibco BRL), PCR buffer, forward and reverse gene primers, and deionized. The primers used for priming the COX-2 gene were as follows: forward, 5′ to 3′, GTGCCTGGTCTGATGATGTATGC and reverse, 5′ to 3′, CCATAAGTCCTTTCAAGGAGAATG. The primers used for priming the internal standard β-actin were, forward 5′ to 3′, CCGCAAATGCTTCTAGGC, and reverse, 5′ to 3′ GGTCTCACGTCAGTGTACGG. PCR bands were identified by size after electrophoresis on a 1% agarose gel in TAE buffer. The gel stained with ethidium bromide, viewed by UV light, and photographed. The bands of interest were photographed scanned using a scanner. The peak areas and densities were determined using NIH Image 1.55 program written by Wayne Rasband at the U.S. National Institutes of Health, Bethesda, MD.

Cell Number: Cell counting was performed using the ZBI Coulter Counter (Coulter Electronics, Inc.; Hialeah, FL) with isotonic buffered saline (Baxter; Deerfield, IL) as blanks.

RESULTS

Changes in the PC-3 Cell Number in Response to PGE_2 and NSAID Administration

The effect of exogenous PGE_2 on the growth of prostatic carcinoma PC-3 cells was investigated. As shown in Figure 1, PC-3 cells grew 2-fold higher from day 0 to 2 . Exogenous PGE_2 at a concentration of 5 μg/ml was able to increase the cell number by approximately 2-fold as compared to the control cultures seen at the end of the 2 day treatment period. The NSAID flurbiprofen, however, reversed the increased in cell number brought about by exogenous PGE_2.

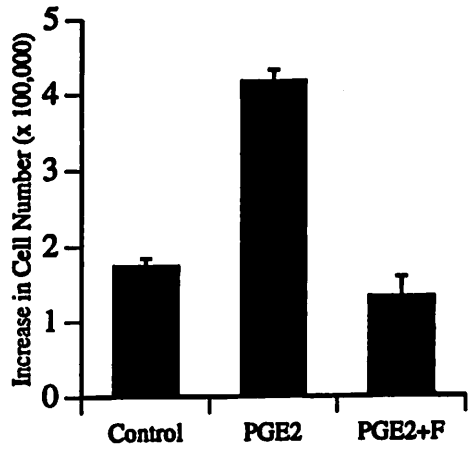

Figure 1. Changes in PC-3 cell number in response to PGE_2 stimulation. PC-3 cells were plated in 6-well plates ($1.2x10^5$ cells/well) in 4 ml of RPMI-1640 medium containing 2 % fetal bovine serum supplemented with antibiotics/antimycotics. The cells were grown for a period of two days in the absence and presence of exogenous PGE_2 (5 μg/ml). Each day the cells were counted increase in the cell number as described in the Experimental Procedure section. The data were presented as an average +/- SD of triplicate treatments.

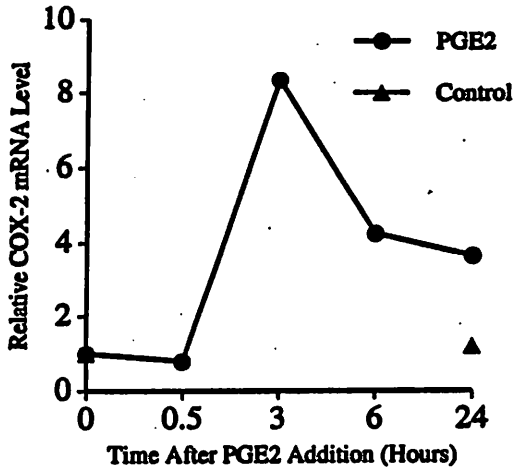

Figure 2. Time course of COX-2 mRNA induction following PGE_2 administration. PC-3 cells were grown in 100 mm culture dishes ($9x10^5$ cells/plate) in RPMI medium containing 0.3 % serum for a period of 48 hours. At time = 0, cells were treated with PGE_2 (5 μg/ml in ethanol). Control culture received ethanol only. Cells were harvested at the indicated time and the RNA was isolated as described in the Experimental Procedure section. The results were presented as the relative level of COX-2 mRNA induction in comparison to the control. The data were representative of 3 experiments.

Time-Dependent Changes in the COX-2 mRNA Levels Following Exogenous PGE_2 Administration

The time course of induction of COX-2 mRNA expression was investigated over a 24 hour period of PGE_2 treatment to PC-3 cells (Figure 2). The steady state COX-2 mRNA began to accumulate somewhere between 1–2 hours following the addition of exogenous PGE_2. At 3 hours, the COX-2 mRNA expression reached its highest level at 8-fold above the level seen at the time of treatment. Beyond 3 hours, the COX-2 mRNA level decreased significantly at the 24 hour time point to 2.5-fold lower than the level seen at 3 hour.

The Effect of Increasing Exogenous PGE_2 Concentration on COX-2 mRNA Level

We investigated the dose-dependent response of exogenous PGE_2 treatment on the steady-state COX-2 mRNA level. As seen in Figure 3 the COX-2 mRNA level was stimulated by 0.5 μg/ml of PGE_2 (1.31 μM) to 2.8-fold higher than the control level. At 5 μg/ml media PGE_2 concentration, the steady-state COX-2 mRNA accumulation was still up-regulated to the same level as that of the 0.5 μg/ml media PGE_2 concentration. However, at 10 μg/ml PGE_2, the COX-2 mRNA accumulation was significantly decreased from the level reached at 5 μg/ml media PGE_2 concentration back to the control level.

The Effect of the NSAID Flurbiprofen on the COX-2 mRNA Accumulation

We set out to investigate whether the induction of COX-2 mRNA is also regulated by the newly-synthesized endogenous PGE_2. Flubiprofen dose-response experiment was

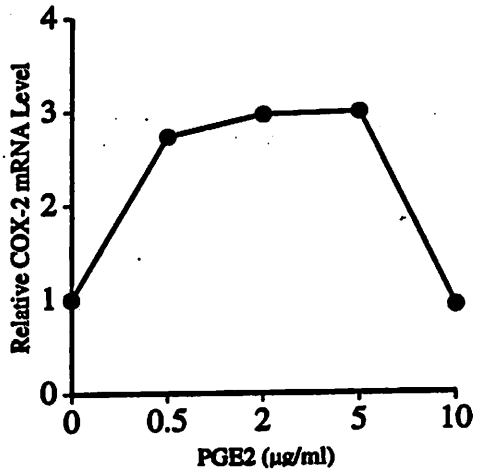

Figure 3. The effect of increasing PGE_2 concentration on COX-2 mRNA level. PC-3 cells were grown and serum depleted in 100 mm culture dishes ($5.5x10^5$ cells/plate) in RPMI medium containing 0.3 % serum for a period of 48 hours. At time = 0, cells were treated with PGE_2 (in ethanol) at the indicated concentration, while the control culture was treated with ethanol only. Cells were harvested following 3 hours of PGE_2 administration and the RNA was isolated as described in the Experimental Procedure section. The results were presented as of the level of COX-2 mRNA induction in comparison to the control. The data were representative of 3 experiments.

carried out to determine the effect of NSAID on COX-2 gene expression. As seen in Figure 4, 1 μM of Flurbiprofen significantly decreased the COX-2 gene expression brought about by PGE_2 to a level 0.75-fold lower than when only PGE_2 was present. The cells treated with 5 μM flurbiprofen, the COX-2 mRNA level reduced even further to 0.90-fold lower than that of the control. This data suggests that the newly-synthesized endogenous PGE_2 is partly responsible for the signal regulating the up-regulation of COX-2 gene expression.

DISCUSSION

We present in this paper evidence suggesting that PGE_2 increases PC-3 cell growth and activates COX-2 gene expression. The up-regulation of COX-2 expression in PC-3 cells depends partly upon new synthesis of PGE_2. Moreover, inhibition of cyclooxygenase with flurbiprofen is able to decrease both growth and COX-2 expression. These facts are interesting since this cell line has previously been shown to be responsive to growth stimulation by an omega-6 polyunsaturated fatty acid, linoleic acid (an essential fatty acid and a PGE_2 precursor). This stimulation is thought to be dependent upon eicosanoid biosynthesis (5). The data presented in this paper provide the first evidence that prostaglandin E_2 acts as a non-polypeptide growth factor in cancerous human cells. Past studies have shown that PGE_2 can act as an autocrine growth factor in bone formation and development both *in vivo* and *in vitro* (10, 11). Furthermore, PGE_2 has also recently been implicated in the growth and differentiation of human B-lymphocytes activated through their CD40 antigen (12).

The administration of NSAID flubiprofen decreased PC-3 cell growth (Figure 1) and the increased COX-2 mRNA level brought about by exogenous PGE_2 (Figure 4). However, it is still inconclusive to us whether this reduction in cell growth and COX-2 mRNA level was due to a direct inhibitory effect of flurbiprofen on COX-2 protein or other indi-

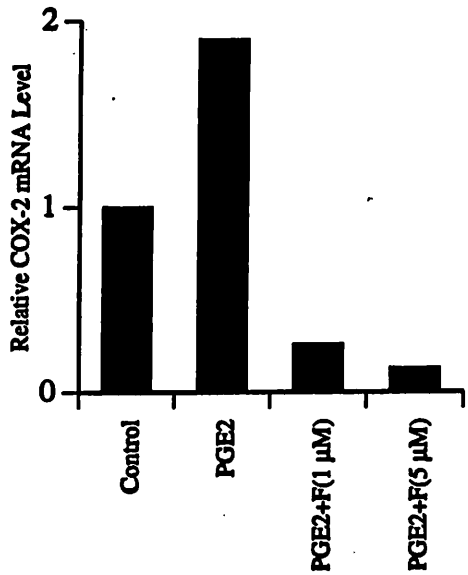

Figure 4. The effect of NSAID Flurbiprofen on COX-2 mRNA accumulation. PC-3 cells were seeded at $6.6x10^5$ cells per plate in 10 mm plate containing 0.3 % serum, and were serum depleted for a period of 48 hours prior to treatment. Cells were treated with PGE_2 (5 μg/ml in ethanol) in the presence or absence of 1 and 5 μM of Flurbiprofen. The control culture was treated with ethanol only. After 3 hours of treatment, the cells were harvested and the RNA was isolated as described in the Experimental Procedure section. The results were presented as the level of COX-2 mRNA induction in comparison to the control. The data were representative of 3 experiments.

rect effects associated with decreased growth gene expressions. However, the results presented in this paper strongly suggest that this reduction is partly due to reduction in the new PGE_2 synthesis by NSAID. It is therefore interesting to speculate whether NSAIDs can potentially be used as chemopreventive agents against the development of prostate cancer, as has been suggested for colon cancer (13, 14).

The cyclooxygenase-2 has recently been classified as an immediate-early genes (2, 15). There are many lines of evidence showing that the gene encoding this enzyme is inducible by varieties of hormones and growth factors. However, the data presented in this paper show for the first time that prostaglandin E_2 up-regulates the expression of its own synthesizing enzyme, COX-2, in a human cancer cell line. The data also suggest that the cells continuously sustain their growth in part by utilizing the extracellular PGE_2 that they themselves produce and release, to up-regulate the expressions of COX-2 as well as other growth genes. Indeed, stimulation of *c-fos* and *Egr-1* expression by arachidonic acid in 3T3 fibroblasts has been found to depend upon PGE_2 formation (16). Moreover, up-regulation of COX-2 expression in PC-3 cells by PGE_2 partly depends upon new synthesis of PGE_2 by the cells (Figures 1 and 4).

The molecular mechanisms behind the activation of COX-2 expression by exogenous PGE_2 has yet to be determined. However, the PGE_2 receptor of the EP-1 subtype, but not EP-2 or EP-3, expression has been detected in the PC-3 cells (data not shown). This finding suggests that a signal transduction involving calcium and possibly, protein kinase C, is playing a role in the activation. Nevertheless, the expression of EP-1 receptor mRNA

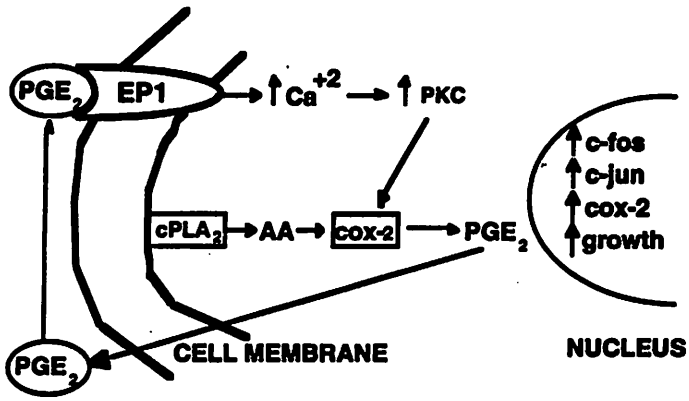

Figure 5. Model of up-regulation of COX-2 by PGE_2 in PC-3 cells.

in the human prostate cells suggests a functional role of EP-1 subtype in mediating the physiological PGE_2 roles in maintenance of prostate cancer homeostasis.

In conclusion, we have showed evidence which suggests that PGE_2 regulates cellular growth and the expression of COX-2 gene in a human prostatic carcinoma cell line. We propose a model (depicted in Figure 5) showing the signal transduction behind the up-regulation of COX-2 in these PC-3 cells. The binding of extracellular PGE_2 to its receptor, EP-1 type, leads to elevation in the intracellular calcium concentrations as well as activation of cellular protein kinase C. These will result in the increase of COX-2 and other growth-related genes (such as *c-fos* and *c-jun*) expression through as yet unidentified mechanism, leading to increased synthesis of COX-2 protein. The newly-made COX-2 enzyme will then synthesized more PGE_2 in which large percentage will be exported out into the extracellular fluid. The cycle starts over with the binding of these newly-synthesized and exported PGE_2 to the EP-1 receptor. The end result of this cycle is growth and proliferation of the cells.

Such regulation seems to be important in the maintenance of growth and homeostasis of the prostate cancer cells, and possibly of normal and cancerous human cells from different tissue origins as well. Since this cell line is responsive to growth stimulation by linoleic acid (5), the fact that its metabolite, PGE_2, stimulates prostate cell growth brings us closer to defining a molecular connection between dietary fat and increased cancer growth.

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