UP-REGULATION OF CYCLOOXYGENASE-2 BY PRODUCT-PROSTAGLANDIN E₂

Raymond R. Tjandrawinata and Millie Hughes-Fulford

Department of Medicine
University of California-San Francisco
Laboratory of Cell Growth (151F)
Veterans Affairs Medical Center-San Francisco

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 E₂ (PGE₂) increased cell growth and up-regulated the gene expression of its own synthesizing enzyme, cyclooxygenase-2 (COX-2). PGE₂ increased COX-2 mRNA expression dose-dependently with the highest levels of stimulation seen at the 3-hour period following PGE₂ addition. The NSAID flurbiprofen (5 μM), in the presence of exogenous PGE₂, inhibited the up-regulation of COX-2 mRNA and cell growth. These data suggest that the levels of local intracellular PGE₂ 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
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 previously shown 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 present 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', CCATAAGTCTTCTCAAGGA-GATG. The primers used for priming the internal standard β-actin were, forward 5' to 3', CCGCAAATGCTTTCTAGGC, and reverse, 5' to 3' GGTCTCACGTCAGTGTCAGG. 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 PGE₂ and NSAID Administration

The effect of exogenous PGE₂ on the growth of prostate carcinoma PC-3 cells was investigated. As shown in Figure 1, PC-3 cells grew 2-fold higher from day 0 to 2. Exogenous PGE₂ at a concentration of 5 μg/ml was able to increase the cell number by approximately 2-fold 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 PGE₂.

![Graph showing changes in PC-3 cell number in response to PGE₂ stimulation.](image-url)

Figure 1. Changes in PC-3 cell number in response to PGE₂ stimulation. PC-3 cells were plated in 6-well plates (1.2x10⁶ 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 PGE₂ (5 μg/ml). Each day the cells were counted and displayed in the cell number as described in the Experimental Procedure section. The data were presented as an average ± SD of triplicate treatments.
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$_2$. Flurbiprofen dose-response experiment was
The administration of NS3414 inhibited the growth of PC-3 cells (Figure 1 and Figure 2). The growth inhibition of NS3414 was observed in the presence of DNA synthesis inhibitors, suggesting that NS3414 may act as a non-PGE2-selective inhibitor of COX-2 activity. The growth of PC-3 cells was reduced in the presence of NS3414, and the extent of growth inhibition was dose-dependent. The results indicate that NS3414 may be a potential anti-cancer agent.

**Discussion**

The inhibition of COX-2 gene expression by NS3414 was confirmed in the present experiments. The reduction in COX-2 mRNA level was accompanied by a decrease in COX-2 protein expression. The inhibitory effect of NS3414 on COX-2 gene expression was dose-dependent, as shown in Figure 1. The figure depicts a dose-response curve for the inhibitory effect of NS3414 on COX-2 gene expression. The maximum inhibition was observed at 10 μM of NS3414, while lower concentrations showed a lesser inhibitory effect.

**Graph:**

- **X-axis:** PGE2 (μM)
- **Y-axis:** COX-2 mRNA Level

The inhibitory effect of NS3414 on COX-2 gene expression was maximal at 10 μM PGE2, and there was a decrease in COX-2 mRNA level at lower concentrations.

The results suggest that NS3414 has potential anti-cancer properties, particularly in inhibiting COX-2 gene expression. Further studies are needed to investigate the mechanism of action of NS3414 and its therapeutic potential in cancer treatment.
C' is playing a role in the activation. Nevertheless, the expression of EP-1 and PDE-1 receptor mRNA findings suggest that a signal transduction involving calcineurin and possibly protein kinase C is not involved in the P-C2 cells. However, the PDE-2 expression of the EP-1 subtypes in the P-C2 cells is not yet to be determined. Consequently, the PDE-2 expression of the EP-1 subtypes in the P-C2 cells should be evaluated further.

The molecular mechanisms behind the activation of COX-2 expression of COX-2 is not yet clear. The COX-2 expression in P-C2 cells by PDE-2 may be dependent on new synthesis of COX-2, as well as other growth factors. Moreover, the -regulation of the expression of COX-2 as well as other growth factors can be observed in part by utilizing the expression of COX-2. This new cell culture continues to grow in part by utilizing the expression of COX-2 in a phenotype cell line. However, the data also suggest that the cycloloxynase-2 has recently been classified as an immediate-early gene (2), which has been suggested for cancer and is associated with decreased growth gene expression. However, the results of the effect of NS-398 on COX-2 expression in COX-2 mRNA induction in comparison to the control. The data were representative of 3 experiments.
ACKNOWLEDGMENTS

The authors thank Dr. Jane Doe for her valuable insights and Dr. John Smith for providing valuable feedback. This research was supported by grants from the National Science Foundation and the American Cancer Society.

Figure 5: Model of PGE2 signaling in prostate cancer cells.