Regulation of Prostaglandin Metabolism by Calcitriol Attenuates Growth Stimulation in Prostate Cancer Cells

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Abstract

Calcitriol exhibits antiproliferative and prodifferentiation effects in prostate cancer. Our goal is to further define the mechanisms underlying these actions. We studied established human prostate cancer cell lines and primary prostatic epithelial cells and showed that calcitriol regulated the expression of genes involved in the metabolism of prostaglandins (PGs), known stimulators of prostate cell growth. Calcitriol significantly repressed the mRNA and protein expression of prostaglandin endoperoxide synthase/cyclooxygenase-2 (COX-2), the key PG synthesis enzyme. Calcitriol also up-regulated the expression of 15-hydroxyprostaglandin dehydrogenase, the enzyme initiating PG catabolism. This dual action was associated with decreased prostaglandin E_2 secretion into the conditioned media of prostate cancer cells exposed to calcitriol. Calcitriol also repressed the mRNA expression of the PG receptors EP2 and FP, providing a potential additional mechanism of suppression of the biological activity of PGs. Calcitriol treatment attenuated PG-mediated functional responses, including the stimulation of prostate cancer cell growth. The combination of calcitriol with nonsteroidal anti-inflammatory drugs (NSAIDs) synergistically acted to achieve significant prostate cancer cell growth inhibition at ~2 to 10 times lower concentrations of the drugs than when used alone. In conclusion, the regulation of PG metabolism and biological actions constitutes a novel pathway of calcitriol action that may contribute to its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and nonselective NSAIDs might be a useful chemopreventive and/or therapeutic strategy in men with prostate cancer, as it would allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects. (Cancer Res 2005; 65(17): 7917-25)

Introduction

In the United States, prostate cancer remains the most common solid tumor malignancy in men, causing ~30,000 deaths in 2005 (1). Effective treatment options include surgery and radiation therapy. The main treatment strategy for advanced prostate cancer involves androgen deprivation therapy to which patients initially respond very well. However, most patients eventually fail this therapy and frequently develop metastatic disease. Current research on prostate cancer aims to identify new agents that would prevent and/or inhibit its progression.

1,25-Dihydroxyvitamin D_3 (calcitriol), the active form of vitamin D, is the major regulator of calcium and phosphate homeostasis in bone, kidney, and intestine (2). However, calcitriol has also been shown to exhibit antiproliferative and prodifferentiation effects in many normal and malignant cells including prostate cancer cells (3–10). There are multiple mechanisms underlying the antiproliferative effects of calcitriol, which vary between target cells (10). These include cell cycle arrest (9, 11) and the induction of apoptosis (12). Several genes that mediate these growth regulatory effects have been identified to be the molecular targets of calcitriol action, such as *p21, p27, bcl-2,* and insulin-like growth factor binding protein-3 (*IGFBP-3*; refs. 5–14). We recently did cDNA microarray analyses to more fully characterize the spectrum of genes regulated by calcitriol in prostate cells (15, 16). Among the newly identified genes regulated by calcitriol, we found two genes which play a key role in prostaglandin (PG) metabolism: the prostaglandin endoperoxide synthase-2 or cyclooxygenase (COX)-2 and the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). PGs are synthesized from free arachidonic acid (17) by COXs. There are two well-characterized COX isoforms: COX-1, a constitutive form of the enzyme, and COX-2, an inducible form of the enzyme. PGs are implicated in the initiation and progression of many malignancies including prostate cancer (18–20). Tumor cells with elevated COX-2 levels are highly resistant to apoptosis, show increased angiogenic potential, and exert suppressive effects on host immunity (19, 20). Nonsteroidal anti-inflammatory drugs (NSAIDs), known inhibitors of both COX-1 and COX-2 enzymatic activity, are under intense investigation to prevent and/or treat malignancies (19, 21). 15-PGDH, which mediates the catalytic inactivation of PGs by converting them to the corresponding keto derivatives, has been found to be down-regulated in some cancers (22, 23) and has recently been regarded as a tumor suppressor gene (24).

In the current study, we investigated the regulation of COX-2 and 15-PGDH by calcitriol in the androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines as well as in primary prostatic epithelial cells derived from normal and cancerous human prostate tissue. Calcitriol reduced the expression of COX-2 and increased that of 15-PGDH. Calcitriol treatment of prostate cancer cells decreased the concentration of prostaglandin E_2 (PGE_2) secreted into the conditioned medium. In addition, calcitriol also decreased the expression of the mRNA of PG receptors EP2 and FP. Our data indicate that these mechanisms led to the attenuation of PG-mediated functional responses by calcitriol, including the suppression of PG stimulation of cell growth. Further, our study showed that the combination of calcitriol and NSAIDs exhibited synergistic growth inhibition, suggesting that the combination might be a useful therapeutic and/or chemopreventive strategy in prostate cancer.

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Materials and Methods

Materials

PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), arachidonic acid, NS-398, and SC-58125 were obtained from Cayman Chemical Co. (Ann Arbor, MI). Calcitriol was a gift from Leo Pharma A/S (Ballerup, Denmark). All stock solutions were made in 100% ethanol and stored at −20°C. Tissue culture media were obtained from Mediatech, Inc. (Herndon, VA). Tissue culture supplements and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Grand Island, NY).

Methods

Cell culture. LNCaP (ATCC no. CRL-1740) and PC-3 (ATCC no. CRL-1435) cells were grown in RPMI 1640 supplemented with 5% FBS, 100 IU/mL of penicillin, and 100 μg/mL streptomycin (Life Technologies). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. Primary cells were derived from radical prostatectomy specimens from men undergoing surgery for prostate cancer treatment. None of the patients had received prior therapy for prostate cancer. The normal cell strains (E-PZ-1 to -3) were derived from peripheral zone tissue with no histologic evidence of cancer in adjacent sections. The cancer cell strains used, E-CA-1 (Gleason grade 3/3), E-CA-2 (Gleason grade 4/5), and E-CA-3 (Gleason grade 4/3), were derived from adenocarcinoma specimens. Primary cell cultures were established from the prostate tissue samples and propagated in culture as we have previously described (25).

Cell proliferation assay. Prostate cancer cells were seeded at an initial density of 1.5×10^5 cells/well in six-well tissue culture plates and allowed to attach overnight in RPMI 1640 with 5% FBS. Cell cultures were shifted to medium containing 2% FBS and treated in triplicate over the next 6 days with either 0.1% ethanol vehicle or the indicated concentrations of drugs. Fresh media and the drugs were replenished every other day. At the end of the treatment, the cells were collected by gentle scraping and subjected to lysis in 0.2 N NaOH. Cell proliferation was assessed by the determination of DNA content (26).

RNA isolation and real-time reverse transcription-PCR. Total RNA was isolated from vehicle or drug-treated cells by the Chomczynski method using Trizol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) as previously described (15). The yield and purity of isolated RNA were checked by UV spectrophotometry. Five micrograms of total RNA were used in reverse transcription reactions using the SuperScript III first strand synthesis kit (Invitrogen). Gene expression in vehicle or calcitriol-treated cells was determined by real-time PCR using the reverse transcription product and gene-specific primers. The reactions were carried out with the DyNamo SYBR Green qPCR kit (Finnzymes, Oy, M.J. Research, Reno, NV) in a 20 μL reaction volume containing gene-specific primers (10 pmol). All real-time PCR reactions were done in duplicate according to the following program: incubation at 72°C for 5 minutes, incubation at 95°C for 5 minutes, and 40 cycles of 94°C for 20 seconds, 58°C for 15 seconds, and 72°C for 20 seconds. PCR products were subjected to agarose gel electrophoresis to determine the purity and size of the amplified products (27). Real-time PCR was carried out using an Opticon 2 DNA engine (M.J. Research). Relative changes in mRNA expression levels were assessed by the $2^{-\Delta\Delta C(T)}$ method (28). Changes in mRNA expression of the different genes were normalized to that of TATA binding protein (*TBP*) gene or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The primers used were as follows: COX-2: 5′-GATACTCAGGCAGAGATGATCTACCC-3′ (sense), 5′-AGACCAGGCACCAGACCAAAGA-3′ (antisense); 15-PGDH: 5′-GACTCTGTTCATCCAGTGCG-3′ (sense), 5′-CCTTCACCTCCATTTTGCTTACTC-3′ (antisense); *c-fos*: 5′-GAATAAGATGGCTGCAGCCAAATGCCGCAA-3′ (sense), 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′ (antisense; ref. 29); EP2: 5′-GTGCTGACAAGGCACTTCATGT-3′ (sense), 5′-TGTTCCTCCAAAGGCCAAGTAC-3′ (antisense); FP: 5′-GCACATTGATGGGCAACTAGAA-3′ (sense), 5′-GCACCTATCATTGGCATGTAGCT-3′ (antisense); *TBP*: 5′-CACTCACAGACTCTCACAACTGC-3′ (sense), 5′-GTGGTTCGTGGCTCTCTTATC-3′ (antisense); *GAPDH*: 5′-GCCTCAAGATCATCAGCA-3′ (sense), 5′-GTTGCTGTAGCCAAATTC-3′ (antisense).

Measurement of prostaglandin E_2 secretion. Subconfluent LNCaP cells were treated with vehicle or calcitriol for 48 hours. Conditioned media were collected and secreted PGE_2 levels were quantitated using a PGE_2 monoclonal enzyme immunoassay kit (Cayman Chemical) according to the protocol of the manufacturer.

Western blot analysis. Cell lysates were prepared from vehicle or calcitriol-treated cells by lysis with a buffer containing 50 mmol/L Tris-HCl, 1 mmol/L EDTA, and 1.6 mmol/L CHAPS (Sigma-Aldrich, St. Louis, MO) supplemented with a protease inhibitor cocktail (Complet, Roche Diagnostics GmbH, Mannheim, Germany). Lysates were incubated at 4°C for 20 minutes and centrifuged at 10,000 × *g* for 1 minute to sediment particulate material. The protein concentration of the supernatant was measured by the Bradford method (30). Proteins were separated in either 10% NuPAGE gels in MOPS-SDS running buffer (Invitrogen) or 10% polyacrylamide Tris-Tricine (Sigma-Aldrich) gels depending on the size of the protein to be detected. After electrophoresis, proteins were transferred onto nitrocellulose membranes by electroblotting. The COX-2 monoclonal (1:1000 dilution) and 15-PGDH polyclonal antibodies (1:250 dilution) used in our study were purchased from Cayman Chemicals. β-Actin monoclonal antibody (dilution 1:500) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Membranes were incubated with the appropriate primary antibodies followed by incubations with a secondary antibody to immunoglobulin G conjugated to horseradish peroxidase (Cell Signaling Technology, Inc., Beverly, MA). Immunoreactive bands were visualized using the enhanced chemiluminescence Western blot detection system (Amersham, Piscataway, NJ) according to the instructions of the manufacturer. The blots were also probed for the expression of β-actin as a control. COX-2 protein was visualized as a ~70 kDa immunoreactive band. 15-PGDH protein was visualized as a ~29 kDa immunoreactive band. Immunoreactive bands were scanned by densitometry (HP Scanjet 7400C) and quantified using Bio-Rad software (Bio-Rad, Hercules, CA). COX-2 or 15-PGDH signals were normalized to β-actin levels in each sample.

Results

We previously showed by cDNA microarray analysis that calcitriol regulated the expression of two of the key genes involved in PG metabolism (i.e., COX-2 and 15-PGDH) in LNCaP human prostate cancer cells (15) and 15-PGDH in primary normal prostatic epithelial cells (16). In the present study, we extended these observations to include an evaluation of calcitriol effects on the expression of these two genes at both the mRNA and protein levels in LNCaP and PC-3 cells. In addition, we also examined the effects of calcitriol in primary prostatic epithelial cell strains derived from normal prostate as well as prostate adenocarcinoma specimens.

Down-regulation of cyclooxygenase-2 expression by calcitriol. Real-time reverse transcription-PCR (RT-PCR) analysis showed significant decreases in COX-2 mRNA levels in both androgen-dependent LNCaP (~70% inhibition) and androgen-independent PC-3 cells (~45% inhibition) due to calcitriol treatment (Fig. 1*A*). Although both LNCaP and PC-3 prostate cancer cells have been shown to express COX-2 protein (31), we found that PC-3 cells exhibited higher basal levels of COX-2 protein expression when compared with LNCaP cells (not shown). We therefore used PC-3 cells to assess the effect of calcitriol on COX-2 protein expression. Figure 1*B* shows that the addition of 10 nmol/L calcitriol to PC-3 cultures for 48 hours reduced COX-2 protein level to ~60% of control, with 100 nmol/L calcitriol having no further effect.

Up-regulation of 15-hydroxyprostaglandin dehydrogenase expression by calcitriol. We examined the effect of calcitriol on 15-PGDH mRNA levels in LNCaP and PC-3 cells. Our data show that 10 nmol/L calcitriol increased 15-PGDH mRNA expression by ~3.6-fold in LNCaP cells and by ~3-fold in PC-3 cells (Fig. 1*C*). We found that the basal protein expression of 15-PGDH varied between different cell lines with the LNCaP exhibiting appreciable levels of the 15-PGDH protein whereas barely detectable levels were seen in PC-3 cells. Therefore, we examined the effect of

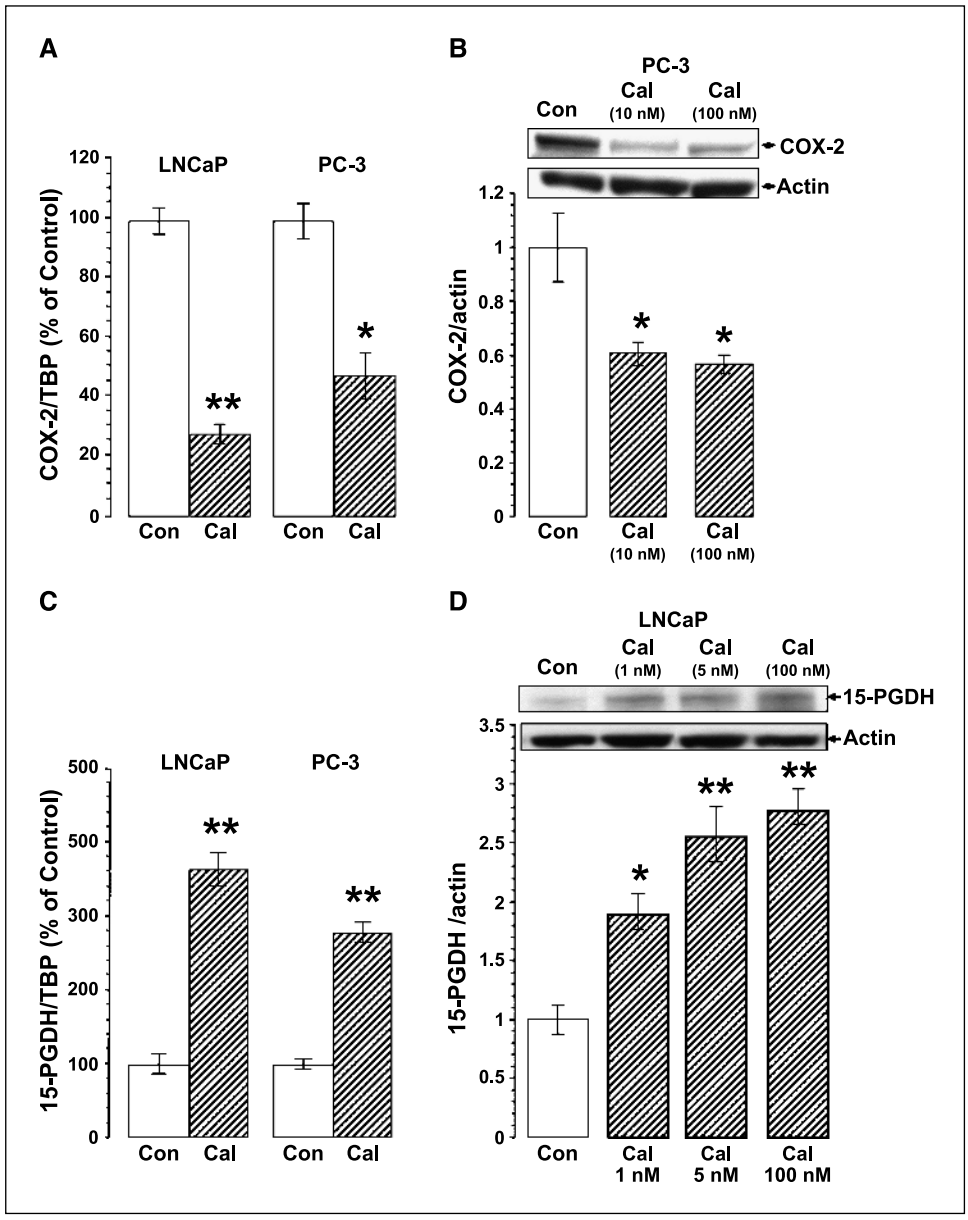

Figure 1. Calcitriol regulates COX-2 and 15-PGDH expression in prostate cancer cell lines. *A,* calcitriol decreases COX-2 mRNA levels. Subconfluent cultures of LNCaP and PC-3 cells were treated with 0.1% ethanol (*Con*) or 10 nmol/L calcitriol (*Cal*) for 24 hours and total RNA was extracted. COX-2 mRNA levels were determined by real-time RT-PCR as described in Materials and Methods and were normalized to *TBP* mRNA levels in the same samples. COX-2/TBP ratio shown as a percent of control set at 100%; *columns,* mean from five experiments; *bars,* SE. *B,* calcitriol decreases COX-2 protein levels. Subconfluent cultures of PC-3 cells were treated with 0.1% ethanol (*Con*) or 10 or 100 nmol/L calcitriol (*Cal*) for 48 hours. Fifty micrograms of total protein were subjected to Western blot analysis as described in Materials and Methods. Representative Western blot. The densitometric units of COX-2 immunoreactive bands were normalized to the densitometric units of the corresponding β-actin bands. Results expressed as the ratio of the control set at 1. *C,* calcitriol increases 15-PGDH mRNA levels. Cells were treated and processed as described in *A*. 15-PGDH/TBP ratio in calcitriol-treated cells given as a percent of control set at 100%; *columns,* mean from five experiments; *bars,* SE. *D,* calcitriol increases 15-PGDH protein levels. LNCaP cells were treated as in *B*. The densitometric units of 15-PGDH immunoreactive bands were normalized to the densitometric units of the corresponding β-actin bands. Results expressed as the ratio of the control set at 1; *columns,* mean of three experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, when compared with control.

calcitriol on 15-PGDH protein expression in LNCaP cells and found a dose-dependent increase in 15-PGDH protein levels in response to calcitriol treatment (Fig. 1*D*).

Calcitriol effects on cyclooxygenase-2 and 15-hydroxyprostaglandin dehydrogenase mRNA levels in primary prostatic epithelial cells. We extended our analysis to include calcitriol effects on primary cultures of prostatic epithelial cells derived from normal prostate as well as adenocarcinoma specimens removed at surgery. Real-time RT-PCR analysis showed considerable decreases (55-90%) in COX-2 mRNA levels in two of the three normal primary cell strains tested (E-PZ-1 and E-PZ-3) after 24 hours of calcitriol treatment (Fig. 2*A*). In all three cancer-derived primary cultures (E-CA-1 to -3) significant reductions (~48-60%) in COX-2 mRNA levels were seen at an earlier time point, after 6 hours of calcitriol treatment, and the down-regulatory effect was lost by 24 hours except in the case of E-CA-2 (Fig. 2*B*). Figure 2*C* and *D* shows the calcitriol-induced changes in 15-PGDH mRNA in primary prostatic cells. In the normal primary cells, calcitriol treatment caused appreciable increases in 15-PGDH mRNA in two of the three strains tested. The time course of this effect showed minor differences. In E-PZ-1 and E-PZ-2 cells significant increases (~2- to 18-fold) were achieved at the end of 6 and 24 hours, respectively (Fig. 2*C*). In two of three of the cancer-derived primary cultures (E-CA-2 and -3), significant increases (~2- to 5-fold) were seen at the end of 24 hours (Fig. 2*D*). In general, the magnitude of COX-2 mRNA down-regulation as well as 15-PGDH mRNA increase was more pronounced in the primary cells derived from normal prostatic tissue when compared with both the cancer-derived primary cells and the established prostate cancer cell lines.

Effect of calcitriol on prostaglandin levels. As a result of the dual action of calcitriol to down-regulate the expression of PG synthesizing COX-2 and increase the PG catabolizing 15-PGDH, we expected a reduction in PG production and secretion in prostate cancer cells treated with calcitriol. We measured the levels of PGE_2 in the conditioned media from LNCaP cells treated with various concentrations of calcitriol for 48 hours. Figure 3*A* shows that the

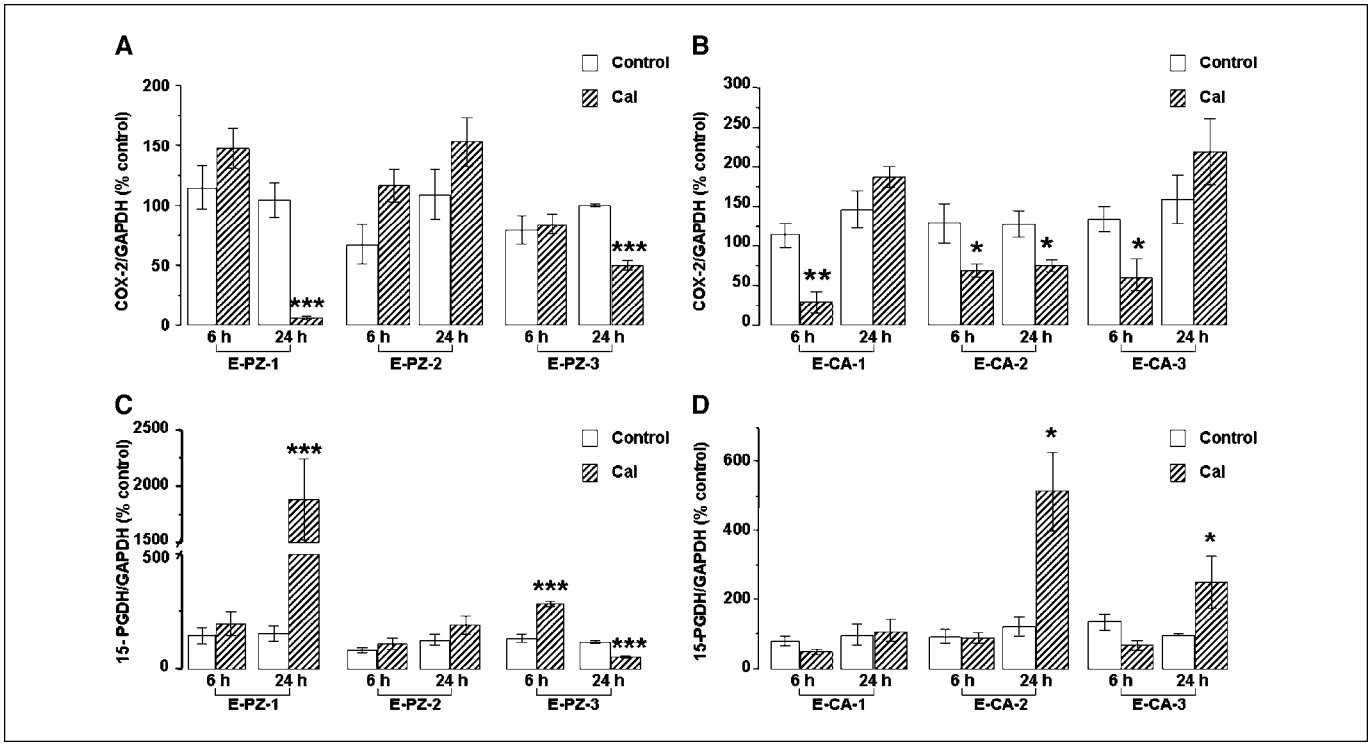

Figure 2. Calcitriol regulates the expression of COX-2 and 15-PGDH mRNA in primary prostatic epithelial cells. Primary cultures of prostatic epithelial cells derived from the peripheral zone of normal prostate tissue (E-PZ-1 to -3) or adenocarcinoma (E-CA-1 to -3) were treated with 0.1% ethanol (*Con*) or with 10 nmol/L calcitriol (*Cal*) for 6 or 24 hours. Total RNA was extracted and COX-2 and 15-PGDH mRNA levels were quantitated by real-time RT-PCR using gene-specific primers as described in Materials and Methods. COX-2 and 15-PGDH mRNA levels were normalized to *GAPDH* mRNA levels and are given as a percent of control set at 100%. *Columns,* mean from three experiments; *bars,* SE. Effect of calcitriol on COX-2 mRNA in three different normal primary epithelial cell strains (*A*) and in cancer-derived cell strains (*B*). Changes in 15-PGDH mRNA in normal cell strains (*C*) and in cancer-derived cell strains (*D*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, when compared with control.

addition of calcitriol caused a significant reduction in PGE_2 secretion with the maximal decrease (~34%) seen with 100 nmol/L calcitriol.

Effects of calcitriol on prostaglandin receptor expression. Prostate cancer cells have been shown to express the PGE receptor subtypes EP2 and EP4 (29). We examined the effects of calcitriol on the expression of the PGE_2 receptor isoforms EP1, EP2, EP3, and EP4, and the $PGF_{2\alpha}$ receptor FP. LNCaP cells treated with 10 nmol/L calcitriol for 24 hours showed a significant (~45%) down-regulation of EP2 mRNA (Fig. 3*B*). We did not detect any changes in the levels of EP1, EP3, or EP4 mRNA following calcitriol treatment (not shown). FP mRNA levels were also down-regulated (~33% decrease) by calcitriol (Fig. 3*C*).

Inhibition of prostaglandin-mediated induction of *c-fos* mRNA by calcitriol. Because calcitriol modulated the levels of biologically active PGs as well as PG receptor expression, we examined its effect on a PG-mediated functional response, (i.e., the induction of the immediate-early gene *c-fos*; ref. 29). As serum is a potent inducer of *c-fos* expression (32), we conducted the experiment under serum-free conditions using PC-3 cells. Unlike LNCaP, PC-3 cells could be briefly maintained in serum-free media for calcitriol pretreatment and subsequent treatment with the PG precursor arachidonic acid. PC-3 cells were pretreated with vehicle or 10 nmol/L calcitriol for 48 hours followed by a brief (30 minutes) exposure to exogenous arachidonic acid (3 μmol/L) directly added to the culture medium. RNA was then isolated and the induction of *c-fos* mRNA was determined as an indicator of the biological activity of PGs endogenously synthesized from arachidonic acid. As shown in Fig. 3*D*, in vehicle pretreated cells arachidonic acid exposure resulted in a significant induction (~2.5-fold) of *c-fos* mRNA levels after 30 minutes. Calcitriol pretreatment completely abrogated the induction of *c-fos* mRNA due to arachidonic acid addition. Calcitriol pretreatment by itself caused a minor increase in *c-fos* mRNA levels when compared with vehicle pretreated cells, which was not statistically significant.

Effects of calcitriol on prostaglandin-mediated growth stimulation. We examined the effect of calcitriol on the stimulation of prostate cancer cell growth by exogenous PG addition as well as by endogenous PGs derived from the substrate arachidonic acid added to the culture medium. We treated LNCaP and PC-3 cells with arachidonic acid (3 μmol/L), PGE_2, or $PGF_{2\alpha}$ (10 μmol/L each) in the absence or presence of 10 nmol/L calcitriol. Our results revealed a moderate but significant growth stimulation by arachidonic acid and exogenous PGs in both LNCaP (Fig. 4*A*) and PC-3 cells (Fig. 4*B*). Calcitriol had a marked growth inhibitory action when given alone. In addition, calcitriol blocked the growth stimulation due to endogenous PGs derived from the added arachidonic acid as well as exogenous PG addition (Fig. 4*A* and *B*).

Synergistic inhibition of prostate cancer cell growth by calcitriol and nonsteroidal anti-inflammatory drugs. We next examined the combined effect of calcitriol and NSAIDs, which are potent inhibitors of COX enzyme activity. We tested a number of both COX-2–selective and nonselective NSAIDs including NS-398, SC-58125, flufenamic acid, sulindac sulfide, indomethacin, naproxen, and ibuprofen. Figure 5*A* to *D* illustrates the effect on prostate cancer cell growth of calcitriol alone

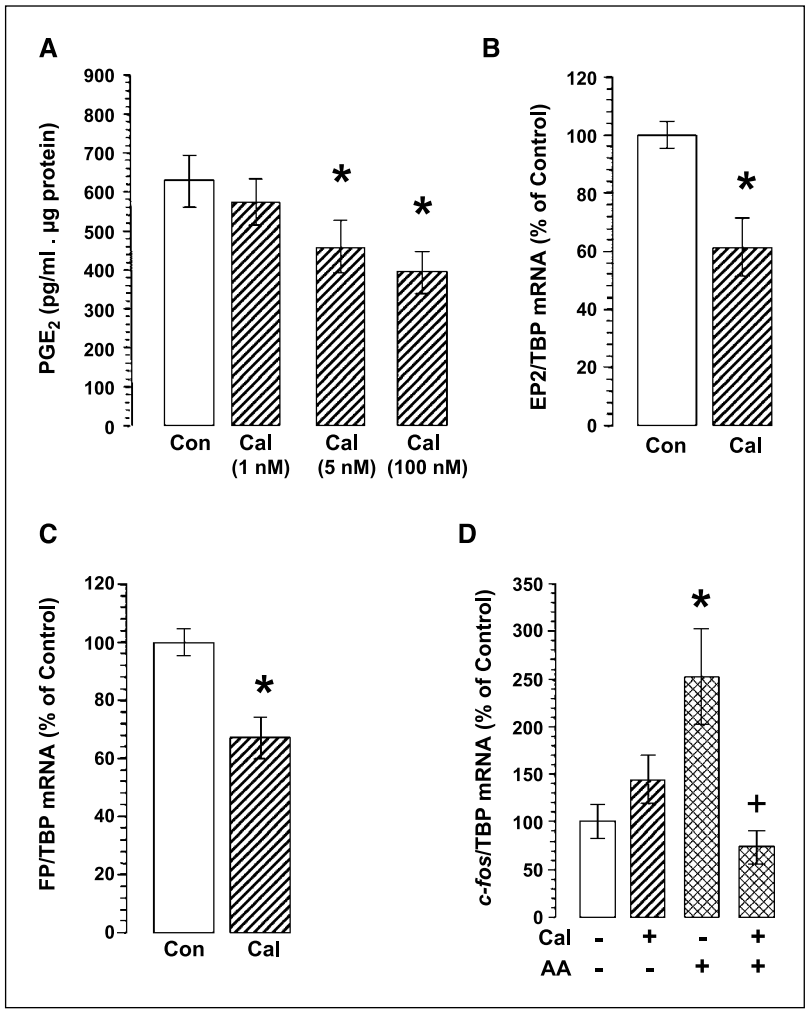

Figure 3. *A,* calcitriol decreases PGE_2 levels. Subconfluent cultures of LNCaP cells were treated with 0.1% ethanol (*Con*) or with the indicated concentrations of calcitriol (*Cal*) for 48 hours. Conditioned media from control and calcitriol-treated cultures were collected and PGE_2 levels were determined using an enzyme immunoassay kit (Materials and Methods). *Columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$. *B,* changes due to calcitriol treatment in EP2 mRNA. LNCaP cells were grown to subconfluence and treated with vehicle (0.1% ethanol; *Con*) or 10 nmol/L calcitriol (*Cal*) for 24 hours. Total RNA was extracted and analyzed for the mRNA expression of EP2 by real-time RT-PCR using gene-specific primers as described in Materials and Methods. EP2 mRNA levels were normalized to the *TBP* mRNA levels. Values given as a percent of control set at 100%; *columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, when compared with control. *C,* changes due to calcitriol treatment in FP mRNA. LNCaP cells were treated and processed as in *B* for the mRNA expression of FP by real-time RT-PCR. FP mRNA levels were normalized to the *TBP* mRNA levels. Values are given as a percent of control set at 100%; *columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, when compared with control. *D,* calcitriol inhibits PG-mediated induction of *c-fos* mRNA. Subconfluent cultures of PC-3 cells were transferred to serum-free RPMI 1640 containing 0.1% ethanol vehicle or 10 nmol/L calcitriol during 48 hours (pretreatment). Following the pretreatment, the cultures were exposed for 30 minutes to arachidonic acid (*AA*; 3 μmol/L) added to the culture medium. The cell cultures were then scraped, RNA was isolated, and *c-fos* mRNA levels were determined by real-time RT-PCR as described in Materials and Methods. *c-fos* mRNA levels were normalized to *TBP* mRNA levels and are given as a percent of control set at 100%. *Columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, when compared with control; +, $P < 0.05$, when compared with arachidonic acid.

or in combination with the NSAIDs that exhibited the best growth inhibitory effect when used at a reduced dose. We show the effects of calcitriol alone and in combination with the COX-2–selective NSAIDs SC-58125 on the growth of LNCaP cells (Fig. 5*A*) and NS-398 on the growth of PC-3 cells (Fig. 5*B*). In LNCaP cells, calcitriol by itself had a modest effect (~20%) at 1 nmol/L but caused significant growth inhibition (~40%) at 10 nmol/L (Fig. 5*A*). The addition of the COX-2–specific inhibitor SC-58125 by itself had a modest effect on cell growth (~20% inhibition), which was not statistically significant, at the concentration tested (5 μmol/L). The combination of 1 nmol/L calcitriol with SC-58125, however, had a more pronounced inhibitory effect (~73% growth inhibition with the combination versus ~20% inhibition with the individual agents), indicating a synergistic interaction between these two drugs to inhibit cell growth. SC-58125 also enhanced the growth inhibition seen with the higher concentration of calcitriol (~80% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone). Similar synergistic growth inhibitory effects were evident in PC-3 cells treated with a combination of calcitriol and the COX-2–selective inhibitor NS-398 (Fig. 5*B*). NS-398, when used alone at 7.5 μmol/L, did not affect the growth of PC-3 cells. However, it enhanced the growth inhibition seen with both 1 and 10 nmol/L calcitriol (~60% inhibition with the combination versus ~20% inhibition with 1 nmol/L calcitriol alone, and ~75% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone).

The growth inhibitory effect of calcitriol was similarly enhanced when combined with nonselective NSAIDs that inhibit the enzymatic activity of both COX-1 and COX-2. The nonselective NSAID naproxen at 200 μmol/L did not inhibit the growth of LNCaP cells (Fig. 5*C*). However, it enhanced the growth inhibition seen with 1 and 10 nmol/L calcitriol (~65% inhibition with the combination versus ~48% inhibition with 10 nmol/L calcitriol alone). Similarly, in PC-3 cells (Fig. 5*D*), the nonselective NSAID ibuprofen at 150 μmol/L enhanced the growth inhibitory effect of calcitriol (~74% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone) whereas it did not affect cell growth when used alone at this concentration.

Based on extensive dose-response analysis (not shown), we calculated the interaction index (γ) using an isobolar method (33) for each drug combination. This analysis indicated a synergistic (superadditive) effect. The data suggested that ~2 to 10 times lower concentration of each drug is needed when used in combination to achieve the same degree of growth inhibition as achieved by the individual drugs.

Discussion

Calcitriol acts by multiple pathways to inhibit the proliferation of prostate cancer cells (5–14). Our study shows that the regulation

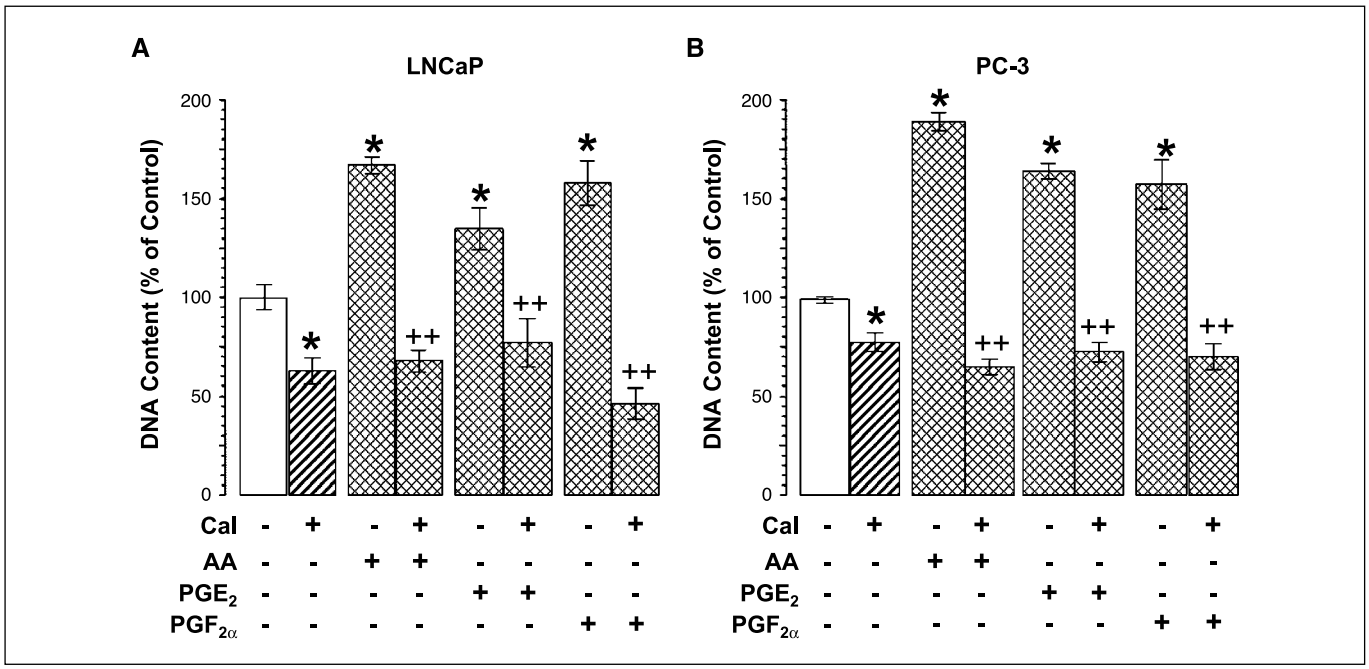

Figure 4. Calcitriol abrogates the growth stimulatory effects of arachidonic acid (*AA*) and exogenous PGs. LNCaP (*A*) and PC-3 (*B*) were treated with arachidonic acid (3 μmol/L), PGE_2 (10 μmol/L), or $PGF_{2\alpha}$ (10 μmol/L) individually or in combination with 10 nmol/L calcitriol (*Cal*) for 6 days. Cell growth was determined by measurement of DNA content as described in Materials and Methods. DNA contents are given as percentage of control value set at 100%, which was equivalent to 12.3 ± 1.2 μg/well for LNCaP cells and 19.3 ± 1.7 μg/well for PC-3 cells. *Columns,* mean from six experiments; *bars,* SE. *, $P < 0.05$, when compared with control; ++, $P < 0.01$, when compared with arachidonic acid, PGE_2, or $PGF_{2\alpha}$ alone.

of PG metabolism is a novel and additional pathway by which calcitriol may exert its antiproliferative actions in prostate cancer cells. We have shown that calcitriol regulates biologically active PG levels and PG actions by three mechanisms: (*a*) the suppression of COX-2 expression, (*b*) the up-regulation of 15-PGDH expression, and (*c*) the reduction of EP2 and FP PG receptor mRNA expression. We propose that these three effects act together to effectively inhibit the stimulation of prostate cancer cell proliferation by endogenously derived PGs as well as PGs added exogenously. Because PGs have been shown to promote prostate cell growth, inhibit apoptosis, and stimulate prostate cancer progression (18–20), we postulate that these effects of calcitriol to reduce PG actions significantly contribute to the anticancer effects of the hormone in prostate cancer.

The transformation of arachidonic acid into PGs and thromboxanes in mammalian cells is catalyzed by the enzyme COX, which has two well-characterized isoforms. COX-1 is constitutively expressed and is involved in housekeeping functions (17, 34). COX-2 is an immediate-early gene that is induced by a variety of growth promoting stimuli such as serum and growth factors, tumor promoters, cytokines, and proinflammatory agents (17, 34), and is regarded as an oncogene (24). COX-2 is overexpressed in various cancers including some, but not all, prostate cancers (18, 21). Inhibitors of COX-2 activity have been shown to suppress prostate cancer cell growth both *in vivo* and *in vitro* (31, 35, 36). Our results show the significant repression of COX-2 mRNA expression by calcitriol in prostate cancer cell lines as well as in primary prostatic epithelial cells and also a reduction in COX-2 protein levels in prostate cancer cell lines, suggesting that COX-2 is a calcitriol target gene.

PGE_2 and $PGF_{2\alpha}$ are rapidly catabolized *in vivo* into their biologically inactive 13,14-dihydro-15-keto metabolites by a two-step process carried out sequentially. The first step is initiated by the reversible oxidation of their 15(*S*)-hydroxyl group by the enzyme 15-PGDH (37). 15-PGDH is widely expressed in many mammalian tissues (38) and has been shown to be modulated by several hormones and factors (37–39), indicating the potential importance of the regulation of this enzyme. In LNCaP cells, 15-PGDH expression is up-regulated by androgens, interleukin-6, and the cyclic AMP inducer forskolin in a protein kinase A–dependent manner (40, 41). We now show that calcitriol is an important regulator of 15-PGDH expression in prostate cancer cells. The partial repression of COX-2 mRNA expression and the increase in 15-PGDH mRNA expression are also seen in primary prostatic epithelial cells derived from normal prostate, suggesting that these calcitriol effects are not restricted to malignant prostate cells. 15-PGDH expression has been shown to be decreased in many cancers (22, 23, 42). Calcitriol has also been shown to increase the expression of 15-PGDH in neonatal monocytes (43), where it exhibits prodifferentiation effects. 15-PGDH, which physiologically antagonizes COX-2, has recently been described as a putative oncogene antagonist that functions as a tumor suppressor in colon cancer by Yan et al. (24) who found that 15-PGDH was universally expressed in normal colon specimens but was routinely absent or severely reduced in cancer specimens. More importantly, stable transfection of a 15-PGDH expression vector into cancer cells greatly reduced the ability of the cells to form tumors and/or slowed tumor growth in nude mice. The authors concluded that 15-PGDH suppressed the effects of the oncogene COX-2 and exhibited an additional effect to inhibit angiogenesis *in vivo* (24). Our present study shows calcitriol-mediated suppression of the oncogene COX-2 and an increase in the expression of the putative tumor suppressor 15-PGDH in prostate cells, suggesting that calcitriol may play an important role in the chemoprevention of prostate cancer.

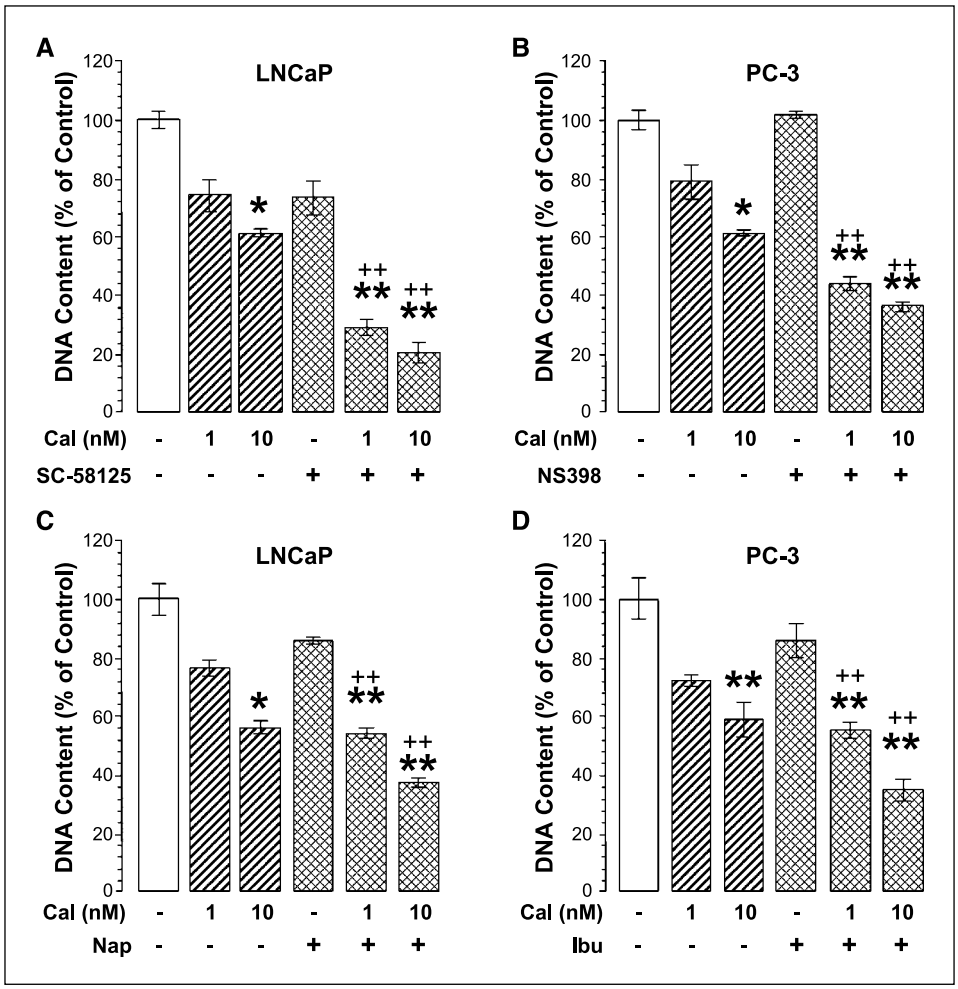

Figure 5. Synergistic inhibition of prostate cancer cell growth by calcitriol and NSAIDs. LNCaP or PC-3 cells were treated with 0.1% ethanol vehicle (*Con*) or 10 nmol/L calcitriol (*Cal*) in the presence and absence of the indicated NSAID. Cell growth was determined by measuring the DNA content as indicated in Materials and Methods. DNA contents are given as percentage of control value set at 100%. *A,* LNCaP cells treated with a combination of calcitriol (*Cal*) and COX-2–specific NSAID SC-58125 (5 μmol/L). 100% DNA content = 10.15 ± 1.22 μg/well. *B,* PC-3 treated with calcitriol (*Cal*) in the presence and absence of the COX-2–selective NSAID NS-398 (7.5 μmol/L). 100% DNA content = 17.42 ± 1.93 μg/well. *C,* LNCaP cells treated with calcitriol (*Cal*) in the presence and absence of the nonselective NSAID naproxen (*Nap*; 200 μmol/L). 100% DNA content = 9.22 ± 0.5 μg/well. *D,* PC-3 cells treated with calcitriol (*Cal*) in the presence and absence of the nonselective NSAID ibuprofen (*Ibu*; 150 μmol/L). 100% DNA content = 21.7 ± 0.9 μg/well. *Columns,* mean from six experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, when compared with control. ++, $P < 0.01$, when compared with 1 or 10 nmol/L Cal alone.

As a result of its dual action to modulate COX-2 and 15-PGDH expression, we expected calcitriol to reduce the levels of PGs in prostate cancer cells. This indeed was the case as shown by the decrease in PGE_2 levels in the conditioned media from LNCaP cells following calcitriol treatment. Calcitriol regulation of PGE_2 synthesis and secretion has been also reported in growth plate chondrocytes (44), in monocytes (43, 45), and in interleukin-1β–stimulated rheumatoid synovial fibroblasts (46). The effects of calcitriol on PG synthesis and signaling in these target cells seem to be related to the rapid nongenomic actions of calcitriol (47).

PGs exert their myriad effects through G-protein coupled membrane receptors which activate different signal transduction pathways (48). Prostate cancer cells have been shown to express the PGE receptor subtypes EP2 and EP4 (29). Interestingly, our study shows that calcitriol decreases the mRNA expression of the PGE_2 and $PGF_{2\alpha}$ receptor subtypes EP2 and FP, providing yet another mechanism for the suppression of the biological activity of PGs by calcitriol. In a recent study examining the changes in gene expression profile in the kidney of vitamin D receptor (VDR) knockout mice, Li et al. (49) report increases in the expression of EP3 and FP genes in $VDR^{-/-}$ kidneys, suggesting that calcitriol may also regulate the expression of PG receptors in kidney. Our study indicates that calcitriol not only decreases the concentration of PGs but may also inhibit the biological activity of these reduced PG levels by repressing of EP2 and FP receptor mRNA expression in prostate cancer cells.

Chen and Hughes-Fulford (29) have shown that arachidonic acid increases the expression of the immediate-early gene *c-fos* by undergoing a COX-2–mediated conversion to PGE_2, binding of PGE_2 to EP2/EP4 receptors, and subsequent activation of the protein kinase A pathway, which leads to the expression of growth-related genes. PGE_2 has also been to shown to up-regulate the gene expression of its own synthesizing enzyme COX-2 in prostate cancer cells, thereby completing a positive feedback loop (31, 50). We therefore examined the effect of calcitriol treatment on the induction of *c-fos* and cell growth by arachidonic acid in prostate cancer cells and found that calcitriol abolished *c-fos* induction and growth stimulation by arachidonic acid. Our interpretation of these observations is that they reflect both the effect of calcitriol to decrease endogenous synthesis of PGs due to COX-2 suppression and the ability of calcitriol to attenuate the biological activity of the PGs due to 15-PGDH up-regulation and EP and FP receptor down-regulation. The suppression by calcitriol of the growth stimulation by exogenous PG addition is probably due to its ability to enhance PG catabolism through the up-regulation of 15-PGDH expression as well as PG receptor down-regulation.

NSAIDs are known inhibitors of COX activity and have been shown to exhibit growth-suppressive effects in *in vivo* and *in vitro* models of prostate cancer (19, 35, 36, 50–52). The growth inhibitory and proapoptotic actions of NSAIDs are due to their ability to inhibit cyclooxygenase activity to a large degree, although in recent years mechanisms independent of COX-2 inhibition are also believed to

play a role (52). Our data show that the combination of calcitriol with COX-2–selective, as well as nonselective NSAIDs, acts synergistically to reduce the growth of prostate cancer cells. Our hypothesis is that the action of calcitriol at the genomic level to reduce COX-2 expression decreases the levels of COX-2 protein and allows the use of lower concentrations of NSAIDs to inhibit COX-2 enzyme activity, resulting in the enhanced growth inhibition seen with the combination. The potential use of NSAIDs as chemopreventive or therapeutic agents for a variety of malignancies, including prostate cancer, is being intensely investigated (20, 21, 51, 53). We propose that a combination of calcitriol and NSAID might be a useful therapeutic strategy in prostate cancer. The clinical use of NSAIDs has recently become controversial because of the cardiovascular complications associated with the use of high doses of COX-2–selective NSAIDs for prolonged periods of time (54, 55). In comparison with the COX-2–selective inhibitors, the use of a nonselective NSAID such as naproxen has been shown to be associated with decreased cardiovascular adverse effects (56). As shown by our study, an enhancement of growth inhibition is seen when calcitriol is combined with nonselective NSAIDs such as naproxen and ibuprofen. The clinical utility of the calcitriol combination with a nonselective NSAID is therefore worthy of evaluation, especially because the combination allows the use of lower concentrations of calcitriol and the NSAIDs, thereby improving the safety profile of the NSAIDs.

In conclusion, calcitriol acts by three separate mechanisms: decreasing COX-2 expression, increasing 15-PGDH expression, and reducing PG receptor mRNA levels. We believe that these actions contribute to suppress the proliferative stimulus provided by PGs in prostate cancer cells. The regulation of PG metabolism and biological actions constitutes an additional novel pathway of calcitriol action mediating its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and a nonselective NSAID, such as naproxen, might be a useful therapeutic and/or chemopreventive strategy in prostate cancer, as it would achieve greater efficacy and allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects.

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