Cytosolic Phospholipase A_2-α: A Potential Therapeutic Target for Prostate Cancer

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Abstract

Purpose: Cytosolic phospholipase A2-α (cPLA$_2$-α) provides intracellular arachidonic acid to supply both cyclooxygenase and lipoxygenase pathways. We aim to determine the expression and activation of cPLA$_2$-α in prostate cancer cell lines and tissue and the effect of targeting cPLA$_2$-α *in vitro* and *in vivo*.

Experimental Design: The expression of cPLA$_2$-α was determined in prostate cancer cells by reverse transcription-PCR, Western blot, and immunocytochemistry. Growth inhibition, apoptosis, and cPLA$_2$-α activity were determined after inhibition with cPLA$_2$-α small interfering RNA or inhibitor (Wyeth-1). Cytosolic PLA$_2$-α inhibitor or vehicle was also administered to prostate cancer xenograft mouse models. Finally, the expression of phosphorylated cPLA$_2$-α was determined by immunohistochemistry in human normal, androgen-sensitive and androgen-insensitive prostate cancer specimens.

Results: cPLA$_2$-α is present in all prostate cancer cells lines, but increased in androgen-insensitive cells. Inhibition with small interfering RNA or Wyeth-1 results in significant reductions in prostate cancer cell numbers, as a result of reduced proliferation as well as increased apoptosis, and this was also associated with a reduction in cPLA$_2$-α activity. Expression of cyclin D1 and phosphorylation of Akt were also observed to decrease. Wyeth-1 inhibited PC3 xenograft growth by ~33% and again, also reduced cyclin D1. Immunohistochemistry of human prostate tissue revealed that phosphorylated cPLA$_2$-α is increased when hormone refractory is reached.

Conclusions: Expression and activation of cPLA$_2$-α are increased in the androgen-insensitive cancer cell line and tissue. Inhibition of cPLA$_2$-α results in cells and xenograft tumor growth inhibition and serves as a potentially effective therapy for hormone refractory prostate cancer.

Previous studies have shown that the eicosanoid pathway is activated in many types of cancers (1) including prostate (2–9). Eicosanoids, which are the products of the cyclooxygenase (COX) and lipoxygenase pathways, contribute to cancer progression by promoting cell proliferation, motility, invasion, and angiogenesis (8–10). Eicosanoids are synthesized from intracellular arachidonic acid, which is released from membrane phospholipids by the action of phospholipase A_2 (PLA$_2$; refs. 11, 12). Of the known mammalian PLA$_2$ enzymes, cytosolic PLA$_2$-α (cPLA$_2$-α), an 85-kDa protein, is the predominant source of intracellular arachidonic acid for eicosanoid production.

We have previously shown that expression of Annexin II, a calcium-dependent phospholipid-binding protein and inhibitory regulator of cPLA$_2$-α, is lost in human prostate cancer (13). We have also reported that secretory PLA$_2$-IIA (sPLA$_2$-IIA), one of the secretory PLA$_2$s, is overexpressed in prostate cancer cells including those remaining after androgen ablation therapy (14). The growth-promoting effect of sPLA$_2$-IIA seems to be via cPLA$_2$-α (14). These data have prompted us to investigate the role of cPLA$_2$-α in prostate cancer growth, and to determine if it is a feasible target for treatment of the hormone refractory stage of prostate cancer.

In this study, we show that cPLA$_2$-α is present in prostate cancer cell lines and increases in androgen-insensitive cells. We also show that small interfering RNA (siRNA) knockdown or specific inhibition of cPLA$_2$-α results in decreased prostate cancer cell growth, through a mechanism of decreased proliferation and, to a lesser extent, increased apoptosis. These effects are at least partially mediated through an inhibition of

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Translational Relevance

The poor response to current therapies by men with hormone refractory prostate cancer highlights the importance of identifying the biological drivers of prostate cancer growth and survival, which may be used as therapeutic targets. Such specific targets have the potential for greater cancer control and significantly less toxicity than current chemotherapy regimens. Identifying that cytosolic phospholipase A2-α (cPLA$_2$-α) is present in prostate cancer cell lines and showing that inhibition of cPLA$_2$-α will suppress cancer cell growth *in vitro* and *in vivo* raise cPLA$_2$-α as a potential therapeutic target. The identification of cPLA$_2$-α and its increase in hormone refractory disease confirms its presence and relevance to human prostate cancer. Our results give evidence that cPLA$_2$-α is a potentially effective therapeutic target and support the need for further studies in the context of clinical trials validating cPLA$_2$-α–targeted therapy for men with prostate cancer.

Akt phosphorylation and a decrease in cyclin D1 expression. We also show that, as seen *in vitro, in vivo*, inhibition of cPLA$_2$-α results in a significant decrease in xenograft tumor growth. Finally, we show that the phosphorylated cPLA$_2$-α (p-cPLA$_2$-α) is present in human prostate tissues and its levels increase in hormone refractory prostate cancer.

Materials and Methods

Reagents and cell lines. The LNCaP-FGC (LNCaP), DU145, and PC3 human prostate cancer cell lines were purchased from the American Type Culture Collection. Cells were maintained in RPMI 1640 (Sigma-Aldrich), supplemented with 10% fecal bovine serum (ICN Biomedical) with all cell cultures at 37°C in a humidified environment of 5% CO_2. The passage numbers of cells described in this article were between 30 and 45 for LNCaP, 65 and 80 for DU145, and 25 and 40 for PC3. The cPLA$_2$-α inhibitor Wyeth-1 was prepared as previously described (ref. 15; U.S. Patent 6,797,708, Sanmar Chemical Company). Pyrrolidine-2 (also known as pyrrophenone) was prepared as previously described (16). Both inhibitors were reconstituted in DMSO. All antibodies [p (Ser^{505})-cPLA$_2$-α, total cPLA$_2$-α, p (Ser^{457})-Akt, total Akt, GAPDH, and α-tubulin] were obtained from Cell Signaling except cyclin D1 (Sigma).

Reverse transcription-PCR. Levels of cPLA$_2$-α mRNA were measured by end-point and real-time reverse transcription-PCR (RT-PCR). After cell treatments, total RNA was isolated using Trizol reagent (Sigma-Aldrich) according to the manufacturer's instructions. The first-strand complementary DNA was synthesized from 2 μg of total RNA using a combination of random hexamers and oligo-dT as described previously (13). Endpoint primers were designed based on the human cPLA$_2$-α mRNA (NM_024420), forward 5′-ACAGTGGGCTCACATTTAACCT, reverse 5′-CTTCCCGATCAAACACATAAGG. GAPDH was used as the housekeeping gene and its primer sequences are forward 5′-TGGACCTGACCTGCCGTCTA, reverse 5′-CCTGTTGCTGTAGCCAAATTC. Conditions for PCR were one cycle of 2 min at 94°C; 40 cycles of 20 s at 94°C, 30 s at 55°C, and 30 s at 72°C; then one cycle of 5 min at 72°C. For real-time PCR, the cPLA$_2$-α primer sequence forward 5′-ATCCTGATGAATTTGAGCGA, reverse 5′-CAAGTAGAAGTTCCTTGAACG. TATA box binding protein (*TBP*) was chosen as the housekeeping gene, forward 5′-GAACCACGGCACTGATTTTC, reverse 5′-CCCCACCATGTTCTGAATCT.

Quantitative PCR measurements were done with SYBR-Green and ROX as a passive reference using the Rotor-Gene system. Conditions for PCR were one cycle of 2 min at 50°C and 2 min at 95°C; 50 cycles of 30 s at 95°C, 30 s at 65°C, and 30 s at 55°C, followed by one cycle for 10 s at 25°C. The Δ-Δ method was used to calculate relative changes in cPLA$_2$-α compared with the housekeeping gene (*TBP*).

Western blotting. Cell lysates were prepared using lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPi, 1 mmol/L β-glycerolphosphate), supplemented with 1:50 dilution of protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was quantified using Bio-Rad Protein Assay (Bio-Rad). Cell lysates (60 μg) were separated on 8% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk containing 0.1% Tween for 1 h. Primary antibodies were incubated overnight at 4°C, washed, and probed with secondary antibodies coupled with peroxide and detected by enhanced chemiluminesence (Amersham Biosciences). Blocking peptide (total and p-cPLA$_2$-α; Cell Signaling) was added at a concentration of 2 μg/mL to the primary antibody, before being added to the blot. NIH-3T3 and HeLa cells were used as positive controls for detection. Densitometric scanning was done to quantify band intensities.

Immunohistochemistry. Prostate cancer cells were scraped with a rubber policeman and fixed in 10% formalin and embedded in paraffin wax. Human prostate tissue was collected from consenting patients, under Central Sydney Area Health Service (X04-0138) and Western Area Health Service Ethics Committee [HREC 2000/9/4.18(1089)] approval. Localized prostate cancer specimens from the peripheral zone of the prostate were obtained from men who had radical prostatectomy as treatment for their prostate cancer (n = 12). Paired prostate cancer specimens before and after reaching hormone refractory stage were also obtained (n = 7 pairs). These men initially presented with prostate cancer, and tissue was obtained when they underwent transurethral resection of prostate. After the cancers progressed and androgen ablation therapy failed, these men required repeat transurethral resection of prostate. Normal prostate tissue from the peripheral zone was also obtained from organ donors as the control.

Cut sections were subject to dewaxing, antigen retrieval, and incubation with primary antibody. Blocking peptide for p-cPLA$_2$-α was added at a concentration of 2 μg/mL to the primary antibody, before addition to the slide. This was followed by the application of biotinylated secondary antibody and Vectastain ABC kit (Vector) and staining revealed using 3,3′-diaminobenzidine (DakoCytomation). The staining intensity was graded as low or high, and the percentage of cells stained at each intensity was recorded.

cPLA$_2$-α gene silencing with siRNAs. The cPLA$_2$-α–specific sequence TTGAATTTAGTCCATACGAAA (Qiagen) and negative control against the nonmammalian gene, fluorescein AATTCTCCGAACGTGTCACGT were used. Prostate cancer cells were transfected with 5 or 10 nmol/L of siRNA duplexes using HiPerfect Transfection Reagent (Qiagen). Transfection efficiency was confirmed by fluorescence microscopy and counting.

Cell growth. The cells were plated in triplicate in 96-well plates. After 48 h, cells adherent on 96-well plates were exposed to the indicated treatments for the stated time intervals. Cell growth was assessed using MTS (CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay; Promega) as described previously (17). Values are expressed relative to untreated controls. Inhibitory concentrations IC$_{25}$, IC$_{50}$, and IC$_{75}$ were calculated by plotting graphs of cell growth measured by MTS versus concentration of drug.

Cytosolic PLA$_2$ activity assay. Arachidonoyl thio-PC was used as the substrate to measure cPLA$_2$ activity *in vitro*. The cPLA$_2$ Assay Kit was purchased from Cayman Chemical and procedures were done according to the manufacturer's instructions. After treatment of cells with Wyeth-1 or siRNA, the cells were homogenized and treated with 7.5 μmol/L bromoenol lactone, a specific independent PLA$_2$ inhibitor. We did not add a sPLA$_2$ inhibitor as it is barely detectable in the PC3 cell line. Twenty microliters of cell lysate were finally subjected to the assay, and the absorbance value was measured at 414 nm and normalized to protein concentration.

DNA synthesis assay. Incorporation of [^{3}H]thymidine was used to measure DNA synthesis. Prostate cancer cells were plated in 96-well plates and allowed to seed for 48 h. The cells were then treated with siRNA or inhibitors. [^{3}H]thymidine (TRK686, Amersham Biosciences) to the equivalence of 1 μCi/100 μL was added for the final 6 h. At the end of treatment, the cells were trypsinized and cellular DNA was harvested onto filtermats using a cell harvester (Perkin-Elmer). Following drying, scintillation fluid was added and radioactivity read in a β plate counter. Counts were normalized to cell number in replicate plates treated the same way.

Apoptosis assay—caspase-3/7. This was quantitated by measuring fluorimetrically activated caspase-3 and caspase-7 with ApoOne caspase-3/7 Homogenous Assay (Promega) and done as per the manufacturer's instructions. Prostate cancer cells were plated onto white 96-well plates (Greiner Bio-One) and after 48 h were treated with siRNA or inhibitors. The caspase substrate and buffer solution were added at the end of treatment and allowed to incubate for 18 h before detection with a Fluroskan Ascent fluorometer (Thermo Labsystems) at excitation wavelength 485 nm and emission wavelength 515 nm. All relative fluorescence units were normalized to cell numbers.

Terminal deoxyribonucleotide transferase–mediated nick-end labeling. The Promega DeadEnd Colorimetric TUNEL System was used to measure the effects of inhibitor on late prostate cancer cell apoptosis and done according to the manufacturer's instructions. Cell blocks were dewaxed, antigen retrieved, and covered with equilibration buffer at room temperature for 10 min followed by 1.5 h incubation in rTdT Reaction Mix at 37°C. The reaction was terminated by immersing slides in 2 × SSC solution and biotinylated nucleotides detected using 3,3′-diaminobenzidine. Positive staining cells were counted and expressed as the average of 10 high-power fields (×40).

PC3 xenografts. PC3 cells (0.5×10^6-1×10^6) were implanted s.c. in the flanks of 6- to 7-wk athymic male nude mice. Mice were randomly distributed into two groups once the tumor size was between 150 and 200 mm^3 (n = 8 mice/group): (a) control-treated with vehicle (DMSO) daily i.p.; or (b) treated with Wyeth-1, 10 mg/kg daily i.p. Tumor growth was assessed twice a week by caliper measurement of tumor diameter in the longest dimension (L) and at right angles to that axis (W). Tumor volume was estimated by the formula, $L \times W \times W \times \pi/6$. Mice were sacrificed when the tumors grew to 1,500 mm^3 (the allowable limit specified by the institution animal ethics committee) and tumors excised for immunohistochemical studies. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Sydney (K14/7-2006/3/4429).

Statistical analysis. All *in vitro* experiments were done in triplicate, and the experiments were repeated at least thrice. The statistical software SPSS version 12.0 was used for analysis. Data were analyzed by Student's t test, χ^2 or ANOVA as appropriate. A two-tailed P value <0.05 was considered significant. Xenograft tumor growth was compared between groups by fitting a repeated measures covariate model (ANCOVA), where the actual time measurements are viewed as a covariate. Results are expressed descriptively as mean ± SE.

Results

Cytosolic PLA$_2$-α is present in prostate cancer cell lines. To determine if cPLA$_2$-α was a potential therapeutic target for the treatment of prostate cancer, we initially determined the expression levels of this enzyme in the three commonly used prostate cancer cell lines. Expression of cPLA$_2$-α mRNA transcripts by end-point RT-PCR (data not shown) revealed that the androgen-sensitive prostate cancer cell line LNCaP expressed far less cPLA$_2$-α mRNA than DU145 and PC3, the two androgen-insensitive cell lines. This was confirmed by quantitative RT-PCR (Fig. 1A; $P < 0.05$). To verify this, protein expression of total cPLA$_2$-α was determined by Western blot analysis of prostate cancer cell lysates and quantitated with densitometric analysis of the protein bands from three separate experiments (Fig. 1B). Immunoblots of total cPLA$_2$-α revealed that LNCaP expressed very small amounts of the protein compared with DU145 and PC3 ($P < 0.05$). As cPLA$_2$-α needs to be activated by phosphorylation, we also determined the level of p-cPLA$_2$-α by Western blot analysis (Fig. 1B). LNCaP again showed very low p-cPLA$_2$-α expression, but the intensity significantly increased for DU145 and even further for PC3 ($P < 0.05$), consistent with the total cPLA$_2$-α expression. We believe antibody binding was specific, as binding was depleted by the addition of excess peptide (Fig. 1C). Further experiments were done only on LNCaP (androgen sensitive) and PC3 (androgen insensitive) prostate cancer cell lines.

To visualize the proportions of cells with active cPLA$_2$-α, cell blocks of LNCaP and PC3 cells were stained by immunocytochemistry with anti-p-cPLA$_2$-α antibody (Fig. 1D). Phosphorylated cPLA$_2$-α stained cytoplasm, perinuclear as well as intranuclear regions. Consistent with the immunoblot findings, LNCaP showed some cells with weak p-cPLA$_2$-α, and PC3 cells had much more intense staining of a larger proportion of cells. Specificity was again confirmed by the addition of a blocking peptide.

Knockdown or inhibition of cPLA$_2$-α results in inhibition of prostate cancer cell growth. To determine if cPLA$_2$-α enzymatic activity is involved in prostate cancer growth, we examined prostate cancer cell growth *in vitro*, after cPLA$_2$-α mRNA was silenced with siRNA. Figure 2A shows that siRNA at 10 nmol/L results in efficient knockdown of cPLA$_2$-α mRNA in LNCaP and PC3 cells at 48 hours, with levels at ~30% to 40% compared with control cells transfected with nonmammalian siRNA. To determine cell growth, cells were treated with siRNA to cPLA$_2$-α for 48 hours and cell number was estimated by the MTS assay. Results show that both LNCaP and PC3 cell numbers were significantly reduced compared with cells transfected with nonmammalian siRNA (Fig. 2B). At doses of 5 nmol/L and 10 nmol/L, siRNA significantly reduced LNCaP cell numbers to 81% ($P = 0.008$) and 70% ($P < 0.001$), respectively, and PC3 cell numbers to 79% ($P = 0.007$) and 65% ($P < 0.001$) respectively.

As specific knockdown of cPLA$_2$-α mRNA resulted in significant reductions in cell growth in both cell lines, we aimed to determine if a newly available specific inhibitor of cPLA$_2$-α named Wyeth-1 by Ni et al. (15) and also named Gfipladib could also reduce prostate cancer cell growth. This compound has been shown not to inhibit sPLA$_2$.[11] LNCaP and PC3 were treated with increasing concentrations of the inhibitor, and growth was measured after 72 hours treatment by MTS assay (Fig. 2C). LNCaP cells showed dose-dependent reductions in cell number ($P < 0.001$) as did the PC3 cell line ($P < 0.001$), but at higher concentrations. The concentration of Wyeth-1 required to achieve a 25% (IC_{25}), 50% (IC_{50}), and 75% (IC_{75}) reduction in cell number was calculated by constructing a line of best fit and applying the resulting equation. The IC_{25}, IC_{50}, and IC_{75} for Wyeth-1 treatment of LNCaP cells were 11.1, 15.9, and 21.0 μmol/L, respectively, and 15.1, 22.2, and 27.6 μmol/L for PC3, respectively. These

[11] M.H. Gelb, unpublished data.

specific Wyeth-1 concentrations were used for further experiments. To determine if the growth inhibitory effects of Wyeth-1 could be repeated using other specific $cPLA_2$-α inhibitors, the experiment was repeated with pyrrophenone-2, another specific $cPLA_2$-α inhibitor. Pyrrophenone also decreased LNCaP and PC3 cell growth in a dose-dependent manner identical to Wyeth-1 except that the inhibitory concentrations were lower (IC_{50} was 3.3 μmol/L for LNCaP and 8.8 μmol/L for PC3). The ability of two structurally different inhibitors, pyrrolidine-2 and Wyeth-1, as well as $cPLA_2$-α silencing, to block cell growth is evidence supporting an important role for $cPLA_2$-α in prostate cancer cell growth.

To further confirm that the inhibitor Wyeth-1 inhibited cell growth by inhibiting $cPLA_2$-α activity, we determined the effect of Wyeth-1 on inhibition of $cPLA_2$-α activity *in vitro*. Activity was quantitated by measuring conversion of a PLA_2 substrate, arachidonoyl thio-PC, to free thiol by $cPLA_2$. Cells were pretreated with an independent PLA_2 inhibitor to ensure all measured hydrolysis was a result of $cPLA_2$ activity. Secretory PLA_2 protein is barely detectable in PC3 cells by Western blot (data not shown). Figure 2D confirms that at IC_{50} Wyeth-1 results in a 41% reduction in $cPLA_2$ activity in PC3 cells ($P < 0.001$). To verify that the reduction in PLA_2 activity was due to $cPLA_2$-α, not any other PLA_2, we also showed a significant reduction in $cPLA_2$-α activity (26%; $P < 0.01$) with knockdown of $cPLA_2$-α mRNA using 10 nmol/L siRNA.

Prostate cancer cell proliferation and apoptosis are regulated by $cPLA_2$-α function. The decreases in cell growth observed with Wyeth-1 treatment could be a result of inhibition of proliferation and/or induction of apoptosis. To determine which processes were involved, we first assessed proliferation by measuring [^{3}H]thymidine incorporation in prostate cancer cell lines after $cPLA_2$-α mRNA silencing with 10 nmol/L siRNA. Figure 3A shows that LNCaP cells and PC3 cells undergo a 28% ($P = 0.04$) and 43% ($P < 0.001$) reduction in proliferation, respectively, 24 hours after treatment with siRNA when compared with cells treated with nonmammalian siRNA. Wyeth-1 treatment at IC_{25} and IC_{50} also resulted in reduced LNCaP cell proliferation by 22% ($P = 0.006$) and 75% ($P = 0.001$), respectively. PC3 cells showed a 50% ($P < 0.001$) and 76% ($P < 0.001$) reduction in proliferation, respectively (Fig. 3A).

Early apoptosis was measured by activated caspase-3/7 assay. Cytosolic PLA_2-α mRNA silencing with siRNA results in a 26% increase in apoptosis in LNCaP cells ($P = 0.03$) and a 19% increase in PC3 cells ($P = 0.02$; Fig. 3B). Wyeth-1 treatment of LNCaP cells showed 82% ($P = 0.03$) and 96% ($P = 0.05$)

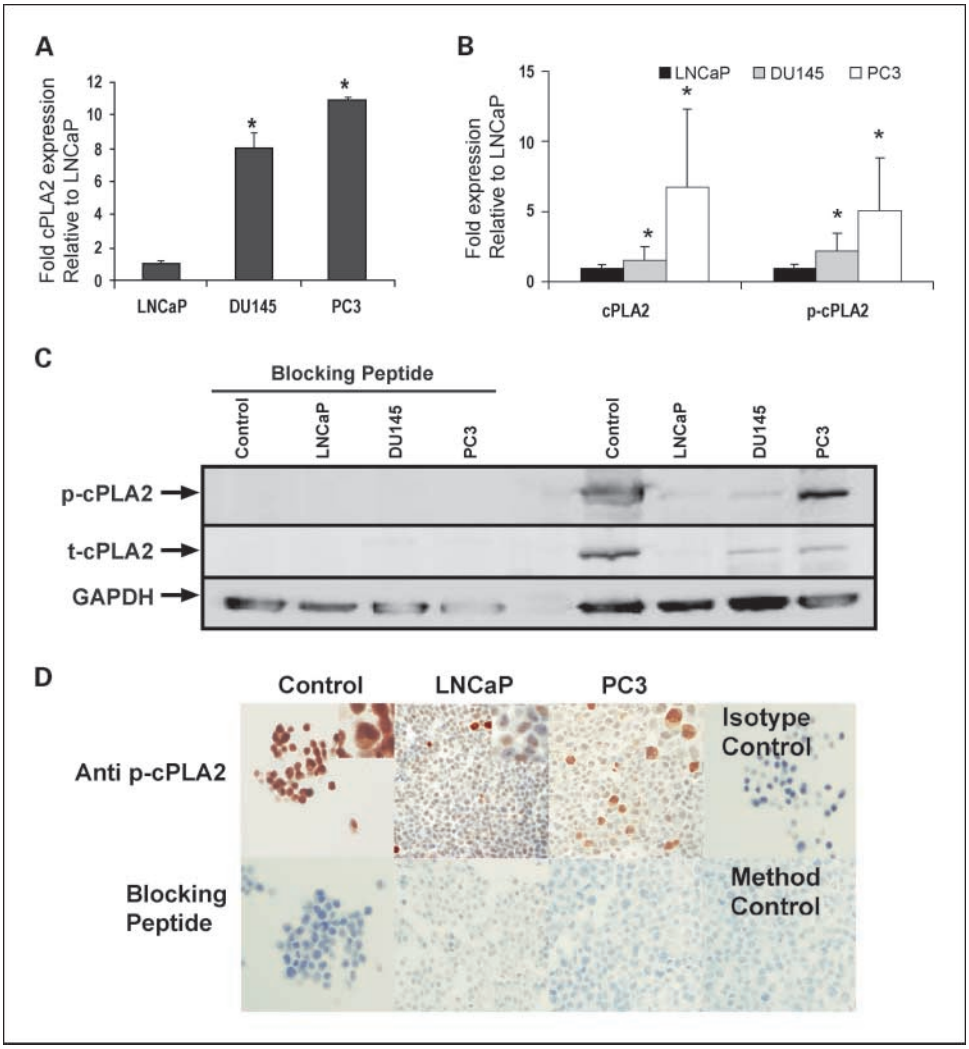

Fig. 1. Cytosolic PLA_2-α is present and levels are higher in androgen-insensitive prostate cancer cell lines. *A,* quantitative real-time RT-PCR from the cell lines (mean ± SD) shows $cPLA_2$-α expression in PC3 › DU145 › LNCaP (*P ‹ 0.05). *B,* staining on immunoblot of cell lines for total and p-$cPLA_2$-α was quantitated by densitometric analysis (mean ± SD) of protein expression on three separate immunoblots (*P ‹ 0.05 compared with LNCaP). *C,* protein levels of total and p-$cPLA_2$-α on immunoblot. Staining on immunoblot was specific for the target protein as bands were absent when the primary antibody was preincubated with the specific blocking peptide. *D,* immunocytochemistry of control HeLa cells show strong staining for p-$cPLA_2$-α, which is abolished with preincubation with blocking peptide. LNCaP and PC3 also show strong staining for p-$cPLA_2$-α. There is little staining with isotype control or method control in either cells line (PC3 shown). All photographs taken at ×40; inserts magnified a further ×4.

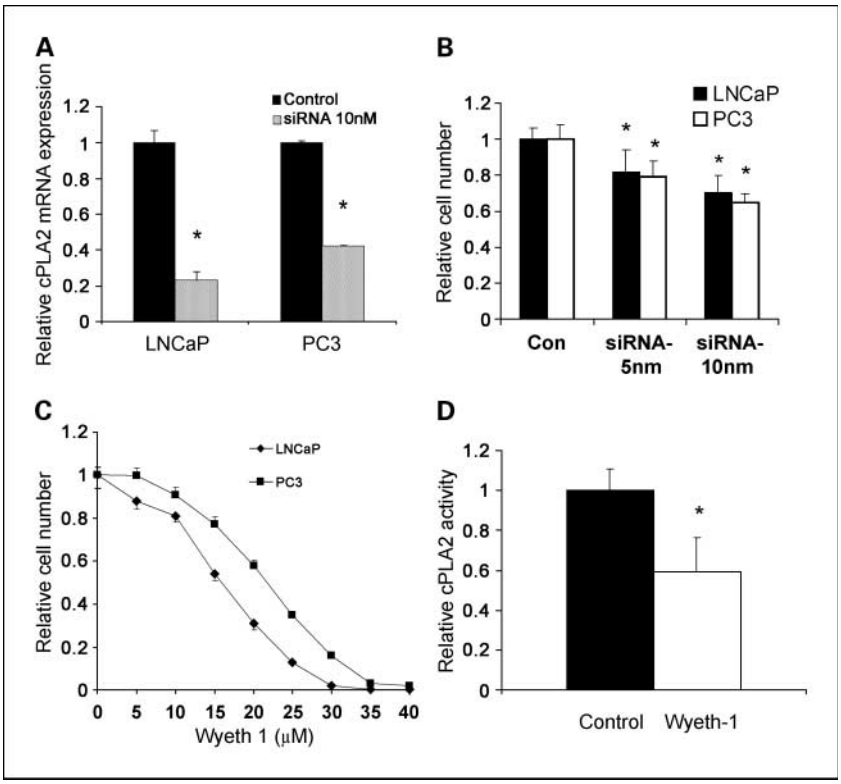

Fig. 2. Cytosolic PLA_2-α gene silencing or inhibition results in decreased prostate cancer cell growth. *A,* $cPLA_2$-α mRNA silencing was achieved with siRNA treatment for 24 h. Quantitative RT-PCR for $cPLA_2$-α show effective knockdown in LNCaP and PC3 cells. *B,* $cPLA_2$-α mRNA silencing after 24 h treatment results in significant reductions in cell number by MTS assay, with increasing concentrations of siRNA in LNCaP and PC3 cell lines (±SD). *C,* inhibition of cell growth by Wyeth-1. Prostate cancer cells were plated onto 96-well plates and treated for 72 h with increasing concentrations of Wyeth-1. Cell growth was measured by MTS assay, and results are expressed relative to control cells (±SD). *D,* inhibition of $cPLA_2$-α activity by Wyeth-1. Cell lysates of PC3 cells treated with DMSO (control) or Wyeth-1 IC_{50} were measured for $cPLA_2$-α activity. Mean activity relative to control were expressed (±SD). * $P < 0.05$ when compared with control.

increases in apoptosis at IC_{25} and IC_{50}, respectively (Fig. 3B). PC3 cells showed a lesser increase at IC_{25} and IC_{50} concentration ($P = 0.02$).

To confirm that the measure of apoptosis by caspase-3/7 activation was also representative of late apoptotic events, we treated LNCaP cells with Wyeth-1 (IC_{50}) for 72 hours and then measured late apoptosis by TUNEL assay of the fixed cell block. Figure 3C shows that compared with control, Wyeth-1–treated cells underwent a 2-fold increase in the percentage of TUNEL-positive cells under high power field.

We conclude that the growth inhibitory effects of $cPLA_2$-α are a result of proliferation reduction and apoptosis induction in both androgen-sensitive and -insensitive cell lines.

Inhibition of $cPLA_2$-α results in a reduction in phosphorylated Akt and expression of cyclin D1. Cyclin D1 is critical for the cell cycle progression, and phosphorylated Akt (p-Akt) is an important part of the phosphatidylinositol 3-kinase-Akt pathway that regulates cell growth and survival. We have previously shown that the treatment of prostate cancer cells with celecoxib, a selective COX-2 inhibitor, results in decreased expression of cyclin D1 (18) and also reduction of p-Akt.[12] We aimed to determine if p-Akt or cyclin D1 were regulated by $cPLA_2$-α. Figure 4A shows that increasing concentrations of Wyeth-1 treatment of LNCaP cells resulted in reductions in p-Akt, particularly at IC_{50} and above. The expression of total Akt was also reduced, but only at concentrations of IC_{75}. Thus, a reduction in total Akt only partially explains the reduction in p-Akt; the remainder must be due to a decrease in proportion phosphorylated.

Wyeth-1 treatment of LNCaP cells also decreased the expression of cyclin D1 at concentrations of IC_{50} or greater (Fig. 4A). To confirm this finding, we did immunocytochemistry for cyclin D1 on cell blocks of LNCaP cells that had been treated with IC_{25}, IC_{50}, and IC_{75} concentrations of Wyeth-1 compound for 72 hours. Figure 4B shows the decrease in nuclear cyclin D1 expression with increasing concentrations of Wyeth-1. Cell counting of cyclin D1-positive cells showed that positive-staining cells decreased from 19% in control to 17% after IC_{25} ($P = 0.31$), 14% after IC_{50} ($P = 0.02$), and 10% after IC_{75} ($P = 0.005$) treatment of cells (Fig. 4C).

Inhibition of $cPLA_2$-α results in PC3 xenograft growth retardation. Based on the *in vitro* findings described above, it was important to investigate whether inhibition of $cPLA_2$-α had comparable growth inhibitory effects *in vivo*. The PC3 xenograft model was used to assess whether Wyeth-1 inhibited tumor growth. Treatment with vehicle or Wyeth-1 (10 mg/kg i.p. daily) was initiated once tumors had formed and reached 200 mm^3 (Fig. 5A). The control (vehicle-treated mice) showed very rapid xenograft tumor growth, and at 14 days of treatment all mice were sacrificed as they had reached the average size of 1,500 mm^3. Comparatively, the Wyeth-1–treated mice showed significantly slower rates of tumor growth. The slopes of the two regression lines, separate for each treatment group, are significantly different ($P < 0.05$). At sacrifice, tumor volumes were 33% smaller compared with control. Mouse health, average weights, and unplanned deaths did not differ between the two groups, suggesting that no major toxicity was suffered as a result of the treatment. Immunohistochemistry for cyclin D1 on xenograft tumors after sacrifice showed considerable reduction in the number of cells staining positive for cyclin D1 in the treated tumors (36%) compared with control tumors (54%; $P = 0.005$;

[12] Unpublished data.

Fig. 5B and C). H&E staining also revealed that control xenograft tumors showed very little necrosis on cross section of the tumor (Fig. 5C) in contrast to the smaller Wyeth-1 – treated xenograft tumors, in which areas of necrosis were seen (Fig. 5C).

Phosphorylated cPLA$_2$-α is increased in human prostate cancer. To determine if cPLA$_2$-α was a potential target in human prostate cancer, we evaluated the expression of p-cPLA$_2$-α in human prostate cancer specimens by immunohistochemistry. As shown in Fig. 6A, prostatic peripheral zone obtained from organ donors without prostate cancer showed weak p-cPLA$_2$-α staining of the normal epithelial compartment of prostatic acini (n = 7). Prostate cancer tissue from radical prostatectomy (Fig. 6B) and transurethral resection of prostate specimens (Fig. 6C) showed slightly higher intensity levels of p-cPLA$_2$-α immunostaining compared with normal. Analysis of the percentage of cells with low or high intensity staining between normal and cancer specimens was not significantly different (P = 0.20). The staining was predominantly localized to the nucleus, but some slides showed weak cytoplasmic staining, consistent with other reported patterns of p-cPLA$_2$-α staining (19).

To investigate whether development of hormone refractory is associated with changes in p-cPLA$_2$-α, we compared the immunostaining of p-cPLA$_2$-α in androgen-sensitive prostate cancer obtained by transurethral resection of prostate (Fig. 6C) with the same patient samples once the patient had reached hormone refractory status and required a repeat transurethral resection of prostate (Fig. 6D). In three of seven paired samples there was a clear further increase in p-cPLA$_2$-α staining intensity in the AI disease status. Illustrated in Fig. 6C and D is one such pair. For the remaining four samples, the staining intensity did not change appreciably with progression of disease. The percentage of cancer cells with low or high intensity staining was significantly higher in hormone refractory status (P = 0.03). We were unable to determine total cPLA$_2$-α staining in tissue specimens using antibodies from Cell Signaling and Santa Cruz for total cPLA$_2$-α as both displayed poor staining immunohistochemically. Figure 6E and F show little background staining on the isotype and method control slides.

Discussion

The arachidonic acid pathway is highly active in prostate cancer (2). Prostaglandin E2, one of the major eicosanoid end-products from the COX arm of the arachidonic acid pathway, results in increased proliferation (20) and decreased apoptosis (21) in prostate cancer cell lines. Inhibition of COX-2 results in decreased prostate cancer cell and PC3 xenograft growth (18, 22). Lipoxygenases, the other arm of the arachidonic acid pathway, produce hydroxyeicosatetraenoic acids (HETE). 5-Lipoxygenase, the enzyme responsible for 5-HETE production,

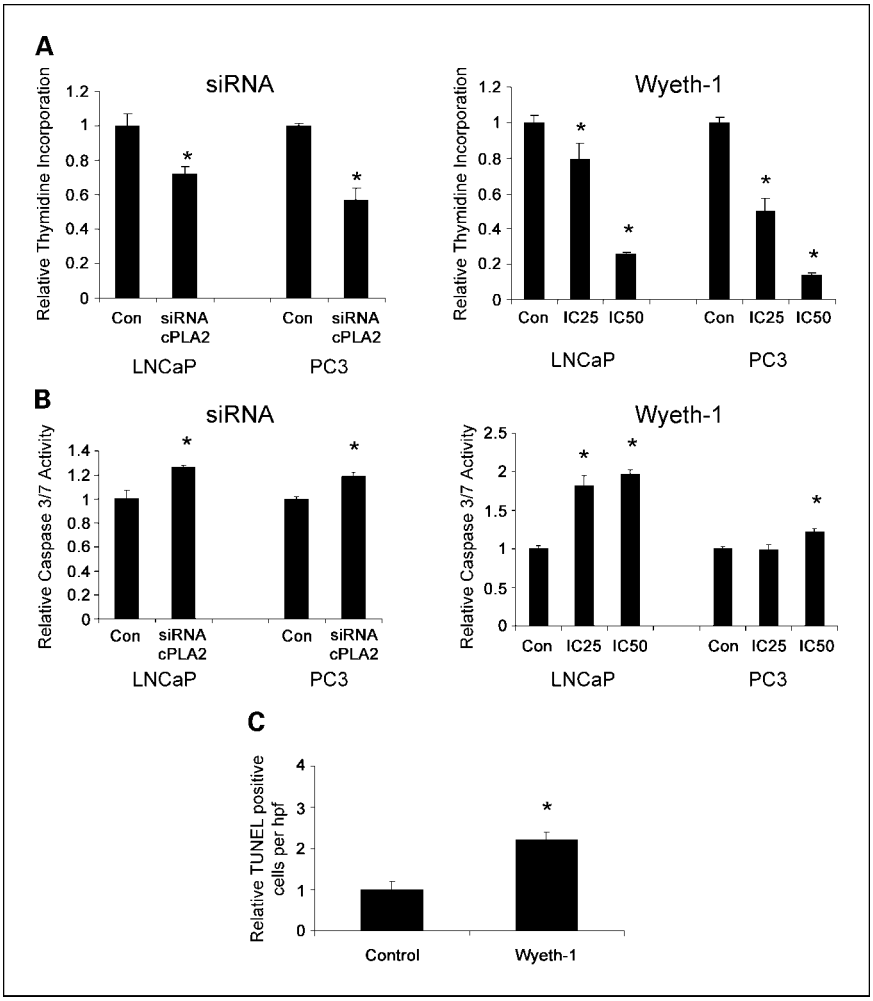

Fig. 3. Cytosolic PLA$_2$-α mRNA silencing or inhibition results in decreased proliferation and increased apoptosis. *A,* prostate cancer cell lines were plated in 96-well plates and treated with siRNA for 24 h or Wyeth-1 at concentrations of IC_{25} and IC_{50} for 72 h. [^{3}H]thymidine was added in the final 6 h. [^{3}H]thymidine incorporation was measured at the end of treatment to quantify DNA synthesis. *B,* prostate cancer cells were treated as in (*A*) and apoptosis measured by a caspase-3/7 assay. *C,* apoptosis as measured by TUNEL. LNCaP cells were treated with IC_{50} Wyeth-1 for 72 h and the cells then fixed and assayed by the TUNEL method. Percentage of stained cells was counted and expressed as the average of 10 high power fields (×40). All results are plotted relative to control cells (±SD).* $P < 0.05$ when compared with vehicle-treated control for the individual cell lines.

is overexpressed in prostate cancer (4), and inhibition of 5-lipoxygenase results in decreased prostate cancer cell proliferation and increased apoptosis (23). 12-Lipoxygenase is also overexpressed in prostate cancer (24), and its product 12-HETE has been shown to increase cancer cell adhesion, migration, and angiogenesis (25). The high activity of both COX and lipoxygenase arms of the arachidonic acid pathway, as well as loss of the natural inhibitor to $cPLA_2$-α (13), led us to hypothesize that $cPLA_2$-α would be a potential target of the treatment of prostate cancer, as inhibition would block a number of very active downstream pathways.

All the human prostate cancer cell lines tested expressed $cPLA_2$-α (Fig. 1). The androgen-sensitive cell line LNCaP expressed considerably less $cPLA_2$-α mRNA and protein compared with the androgen-insensitive DU145 and PC3. A proxy for $cPLA_2$-α activity, p-$cPLA_2$-α, was correlated with the total $cPLA_2$-α levels.

Increased expression of $cPLA_2$-α in prostate cancer cell lines is an interesting phenomenon with a currently unknown mechanism. Cytosolic PLA_2-α is part of the inflammatory cascade and can be induced by tumor necrosis factor-α (26), interleukin-1 (27), and nuclear factor-κB (28, 29). The nuclear factor-κB pathway has been shown to be more active in androgen-insensitive prostate cancer cells compared with androgen-sensitive prostate cancer cell lines, and may be a potential mechanism of $cPLA_2$-α up-regulation (30). In addition, Huges-Fulford et al. have shown that arachidonic acid added exogenously to PC3 cells can up-regulate the expression of $cPLA_2$-α via the production of prostaglandin E2 (28). This is of great importance as dietary intake of fat has been correlated with increased incidence and mortality (31) as well as prostate cancer growth (32). Based on this, Hughes-Fulford et al. have hypothesized that there is a potential for prevention or treatment by dietary reduction of fatty acids or drug inhibition of $cPLA_2$-α or COX (28).

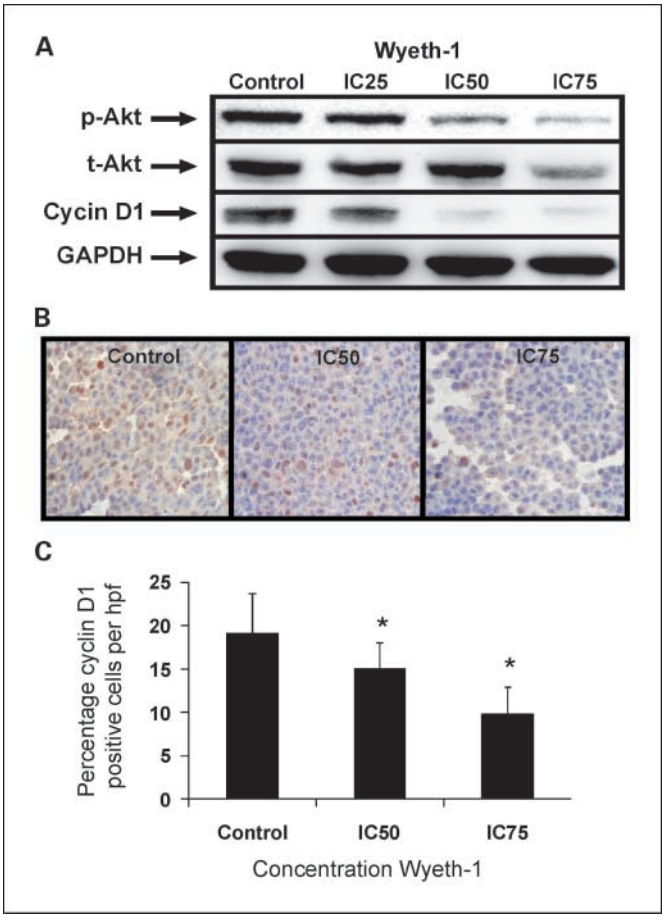

Fig. 4. Cytosolic PLA_2-α inhibition reduces cyclin D1 and Akt expression in prostate cancer cells. *A,* LNCaP cells were treated with vehicle (control) or increasing concentrations of Wyeth-1 for 24 h. Cellular lysate protein (50 μg/lane) was analyzed by Western blot. Reduced levels of p-Akt and cyclin D1 were detected at concentrations of IC_{50}. Reduced levels of total Akt were detected at IC_{75}. *B,* LNCaP cell was treated with increasing concentrations of Wyeth-1. The cell was then paraffin-fixed and stained for cyclin D1. LNCaP exhibited reduced numbers of cells positive for cyclin D1 with increasing Wyeth-1 concentrations. *C,* the percentage of cyclin D1-positive cells with increasing concentration of Wyeth-1. $^*P < 0.05$.

There are several potential mechanisms by which $cPLA_2$-α activity may be aberrantly regulated in prostate cancer cells. First, loss of inhibitory regulators Annexin I and Annexin II occurs in prostate cancer (13, 33). Second, increased mitogen-activated protein kinase activity observed in prostate cancer (34) could be responsible for the phosphorylation-activation of $cPLA_2$-α (35). Third, $sPLA_2$-IIA, which is overexpressed in human prostate cancer (14), could contribute to the phosphorylation of $cPLA_2$-α. In human kidney mesangial cells, $cPLA_2$-α has been shown to be activated by $sPLA_2$-IIA (36) and in a human astrocytoma cell line, $sPLA_2$-IIA has been shown to bind to $sPLA_2$-IIA cell surface receptors, which then stimulate the mitogen-activated protein kinase pathway (37). The activated mitogen-activated protein kinase pathway in turn phosphorylates $cPLA_2$-α, leading to its activation and arachidonic acid production (37, 38).

To determine if $cPLA_2$-α represented a potential target for the treatment of prostate cancer, we initially silenced $cPLA_2$-α mRNA and found a significant reduction in prostate cancer growth (Fig. 2). We then treated the prostate cancer cells with a specific inhibitor of $cPLA_2$-α (Wyeth-1) and showed a dose-dependent inhibition of prostate cancer cell growth. PC3 exhibited more resistance to the effects of Wyeth-1, despite expressing much more $cPLA_2$-α. This may reflect the more aggressive nature of this cell line, which may harbor more active survival pathways. To confirm that the effects of Wyeth-1 were not the result of $cPLA_2$-α–independent effects, we tested the prostate cancer cells with another specific $cPLA_2$-α inhibitor, pyrrophenone (39), and observed similar results. We also showed that Wyeth-1 and $cPLA_2$-α gene silencing effectively inhibit the $cPLA_2$-α activity in prostate cancer cells (Fig. 2D).

To understand the mechanism of proliferation inhibition (Fig. 3A), we elected to study pathways known be involved in prostate cancer. Akt, also known as protein kinase B, is activated by phosphorylation on the membrane. Once phosphorylated, it travels to the cytosol and phosphorylates a variety of target proteins, leading to an increase in cell proliferation and survival. Consistent with other reports (40), we have shown that Akt is strongly phosphorylated in prostate cancer cells, and that Wyeth-1 treatment results in reduced p-Akt (Fig. 4). This mechanism may be mediated by $cPLA_2$-α–facilitated eicosanoid production, as studies of vascular smooth muscle cells show that either arachidonic acid, 5(S)-, 12(S)-, 15(S)-, or 20-HETE addition will increase Akt phosphorylation. Prostaglandin E2 may not be the mediator of Wyeth-1 effect, as inhibition of Akt phosphorylation caused by celecoxib treatment has been shown to be independent of COX-2 in prostate cancer cells (41). A decrease in p-Akt may be a

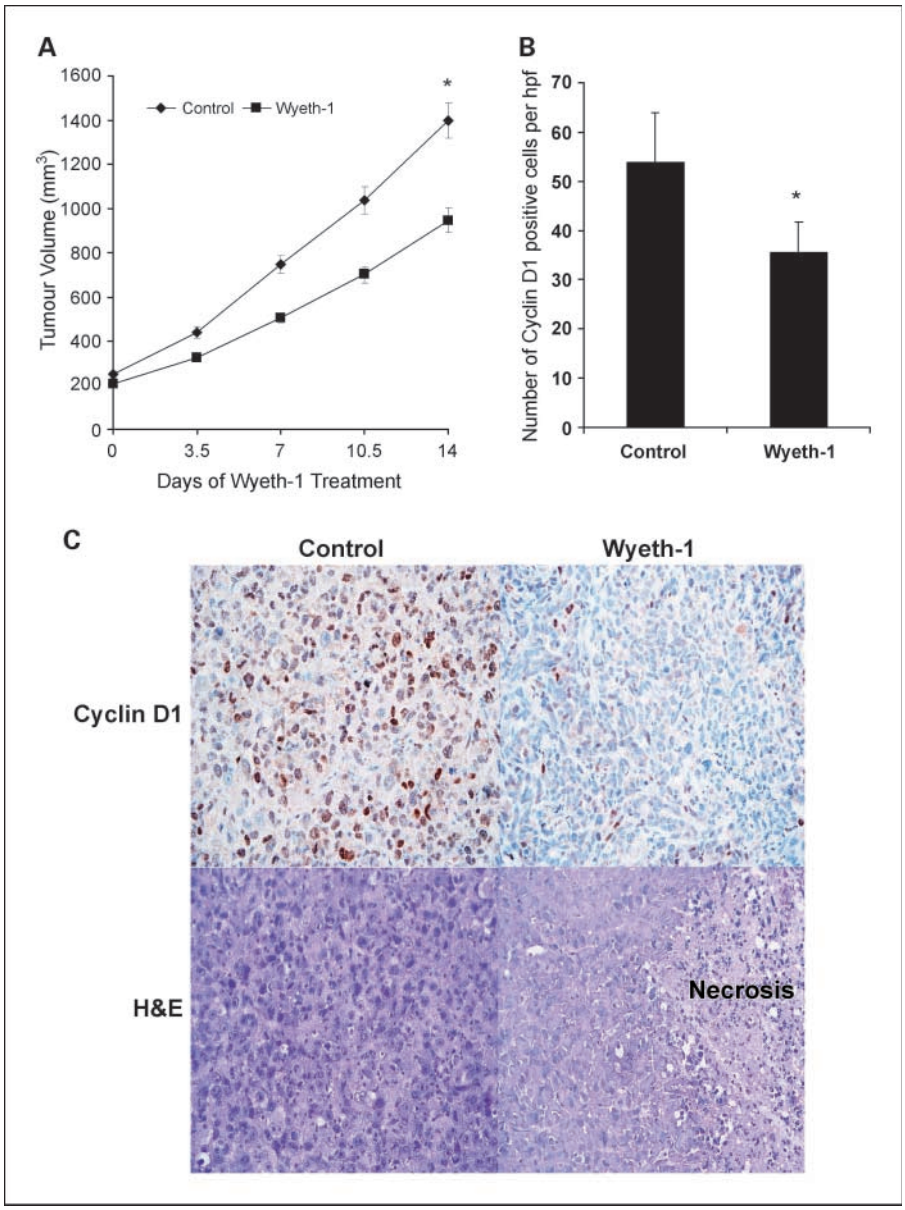

Fig. 5. Cytosolic PLA_2-α inhibition impedes the growth of PC3 xenografts. *A,* PC3 cells were inoculated into the flanks of nude mice. When established xenograft tumors had reached 200 mm^3 in size, the mice were randomized to control (vehicle) or Wyeth-1 treatment, given i.p. daily at a dose of 10 mg/kg (8 mice/group). Statistically significant inhibition of tumor growth was achieved in the Wyeth-1 – treated mice compared with the control animals (mean ± SE; $^*P < 0.05$). *B,* at the time of sacrifice, tumors were harvested and fixed in formaldehyde and paraffin-embedded. Control tumors showed significantly more cyclin D1-positive cells than did Wyeth-1 – treated cells. ($^*P < 0.05$). *C,* the tumors in control and Wyeth-1 – treated mice stained for cyclin D1 and H&E. Tumors from Wyeth-1 – treated mice stained significantly less for cyclin D1 than control. H&E staining showed very little necrosis in control tumors but striking necrosis in the Wyeth-1 – treated tumors.

mechanism by which proliferation is decreased and apoptosis increased after $cPLA_2$-α inhibition.

We have previously shown that celecoxib will decrease expression of cyclin D1 (one of the downstream effectors of Akt pathway) and result in an accumulation of cells in G_0-G_1 with a corresponding decrease in S phase in LNCaP and PC3 cells (18). Consistent with those findings, we found that cyclin D1 expression decreased with Wyeth-1 treatment in LNCaP cells. This also resulted in an accumulation of cells in G_0-G_1 and a corresponding decrease in S phase on flow cytometry (data not shown). It is not yet certain, however, that this is a result of decreased prostaglandin E2 production, as we have previously shown that celecoxib's effect on cyclin D1 could be independent of COX inhibition (18).

As the ultimate aim is to find new treatments for men with high-volume hormone refractory prostate cancer, we chose to use the PC3 xenograft model, and started treatment at a high tumor volume (200 mm^3) to mimic the clinical disease. Despite the large initial sizes of the tumors, Wyeth-1 significantly reduced the rate of growth of tumors by ~33% compared with vehicle-treated mice (Fig. 5). In addition, not only were the Wyeth-1 – treated tumors smaller, but substantial volumes of the tumors were necrotic, unlike the larger control tumors, suggesting that actual viable cancer volume would be even smaller. The cause of the necrosis is not clear but may be due to an inhibition of angiogenesis by $cPLA_2$-α inhibition, as has been shown by COX-2 inhibitors (18, 22); this will be an area of further study in our laboratory. Based on our *in vitro* findings, levels of cyclin D1 were measured in xenograft tumor samples. Similar to what was observed in cultured LNCaP cells, Wyeth-1 caused a reduction in cells staining for cyclin D1 in PC3 xenografts, a finding that has been observed in PC3 xenografts treated with celecoxib (18).

It was important to determine whether the target $cPLA_2$-α was present and active (phosphorylated) in human prostate cancer as this is ultimately where the therapy will be used. In addition, we

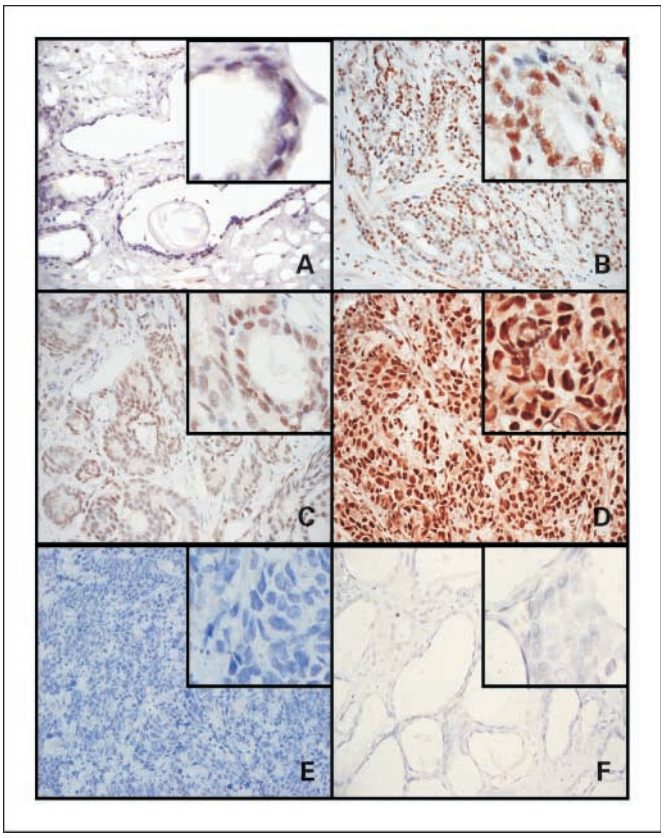

Fig. 6. Activity of cPLA$_2$-α in prostate tissues by immunohistochemical staining of p-cPLA$_2$-α. *A,* normal peripheral zone prostate samples exhibited low levels of phosphorylation of cPLA$_2$-α in epithelial elements, and no staining of stromal tissue. *B,* localized prostate cancer samples from radical prostatectomy exhibited slightly stronger cPLA$_2$-α phosphorylation. *C,* patients who initially underwent transurethral resection of prostate and diagnosed with prostate cancer show the same staining pattern as in (*B*). *D,* after androgen deprivation, some patients who became hormone refractory exhibited increases in p-cPLA$_2$-α staining compared with before androgen deprivation (3 of 7 samples). *E,* isotype control and (*F*) method control show no background staining. All photographs at ×40 magnification. The inserts were magnified (×200) to view nuclear staining.

wanted to confirm whether men with hormone refractory prostate cancer gained increased expression of p-cPLA$_2$-α compared with the androgen-sensitive disease. We are unable to report on expression levels of total cPLA$_2$-α as the immunohistochemical staining of total cPLA$_2$-α, with all available commercial antibodies, was of poor quality in human prostate tissue. We have shown that phosphorylation of cPLA$_2$-α is present in prostate cancer tissue (Fig. 6) and levels may not be much higher in cancer compared with normal tissue; but the levels increased significantly in three of the seven men who developed hormone refractory prostate cancer (P = 0.03). Although this study was small in numbers, the expression of p-cPLA$_2$-α was determined in prostate cancer tissue before androgen ablation, and the tissues from the same men were examined when they reached hormone refractory status; thus, each man acted as his own control. Collectively, the immunohistochemical analyses show that the target enzyme is present and could be higher in some men reaching hormone refractory state.

Although we have shown that cPLA$_2$-α inhibition will decrease cPLA$_2$-α activity and prostate cancer cell growth, we have not investigated another effect of cPLA$_2$-α inhibition such as reduction in platelet activating factor. In cancer cells and hematopoetic cells, platelet activating factor can be produced from the remaining lysophosphoplipid after arachidonic acid cleavage by cPLA$_2$-α. Platelet activating factor has been shown to stimulate nuclear factor-κB as well as angiogenesis (42), and has been shown to be oncogenic in a number of cancers (42–44). Its role in prostate cancer has not been well studied.

A number of other cancers have also been noted to show elevated levels of cPLA$_2$ with the potential for therapeutic targeting (12). In colon cancer, however, the reported actions of cPLA$_2$-α have been controversial. Studies have found that cPLA$_2$-α expression is increased in colorectal cancer (45, 46), but in the Apc^{min} mouse model of colon tumorigenesis, the deletion of cPLA$_2$-α did not result in a change in the number of colon tumors observed (47). Similarly, in azoxymethane-induced mouse colon cancer, cPLA$_2$-α was observed to decrease (48), and when cPLA$_2$-α was deleted in this mouse model, an increase in colon tumors was observed (49). It has been suggested that a loss of arachidonic acid–derived ceramide, a proapoptotic mediator, may result in increased tumor formation in this disease.

Before human phase I studies, further research needs to be done in the area of cPLA$_2$-α inhibition and prostate cancer. Initially, as the class of specific cPLA$_2$-α inhibitors is quite new, pharmacokinetic studies need to be done so proper dosing schedules can be calculated. Further studies in animal models need to look at the effect of varying doses and different stages of disease, such as chemoprevention.

In summary, total and phosphorylated cPLA$_2$-α are present in prostate cancer cell lines. These levels are increased further in androgen-insensitive prostate cancer cell lines. Cytosolic PLA$_2$-α plays a role in cancer cell proliferation and apoptosis, and its inhibition or knockdown results in decreased cell growth. The drug Wyeth-1 seems to act specifically through the cPLA$_2$-α enzyme and not through an off-target effect. Some of these effects can be explained by Wyeth-1–mediated inactivation of Akt and reductions in cyclin D1. These findings are replicated *in vivo*, with decreased PC3 xenograft growth with Wyeth-1 treatment and resulting decrease in cyclin D1 staining of remaining cancer cells. Cytosolic PLA$_2$-α is a potentially effective target for therapy of prostate cancer, and therefore warrants further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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