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Oncogenic Action of Secreted Phospholipase A_2 in Prostate Cancer

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ABSTRACT

Mortality from prostate cancer is associated with progression of tumors to androgen-independent growth and metastasis. Eicosanoid products of both the cyclooxygenase (COX) and lipoxygenase (LOX) pathways are important mediators of the proliferation of prostate cancer cells in culture and regulate tumor vascularization and metastasis in animal models. Pharmacologic agents that block either COX or LOX products effectively reduce the size of prostate cancer xenografts. Phospholipase A_2 (PLA_2) enzymes regulate the provision of arachidonic acid to both COX- and LOX-derived eicosanoids, and a secreted form of the enzyme ($sPLA_2$-IIA) is elevated in prostate cancer tissues. Here, we show by immunohistochemistry, in patients receiving androgen ablation therapy, that $sPLA_2$-IIA remains elevated in remaining cancer cells relative to benign glands after treatment. Furthermore, $sPLA_2$-IIA expression seen in benign glands is substantially decreased after androgen depletion, whereas cytosolic PLA_2-α ($cPLA_2$-α) levels are unchanged. $sPLA_2$-IIA mRNA expression is detectable and inducible by androgen (0.01–10 nmol/L) in the androgen-sensitive cell line LNCaP, and exogenous addition of $sPLA_2$-IIA (1–100 nmol/L), but not an inactive $sPLA_2$-IIA mutant ($H_{48}Q$), results in a dose-dependent increase in cell numbers or the fraction of cells in G_2-M phase, which is inhibited by $sPLA_2$-IIA–selective inhibitors. The effect of exogenous $sPLA_2$-IIA can also be blocked by inhibition of $cPLA_2$-α, suggesting a role for $cPLA_2$-α in mediating $sPLA_2$-IIA action. $sPLA_2$-IIA inhibitors suppressed basal proliferation in LNCaP cells and in the androgen-independent, $sPLA_2$-positive cell line PC3 but not in the $sPLA_2$-IIA–negative androgen-independent cell line DU145. Established PC3 xenograft tumors grew more slowly in mice treated with $sPLA_2$-IIA inhibitors than those treated with saline only. The PLA_2 enzymes, and $sPLA_2$-IIA in particular, thus represent important targets for the treatment of $sPLA_2$-IIA–positive androgen-independent prostate cancer.

INTRODUCTION

Previous studies have demonstrated that the eicosanoid pathway is activated in prostate cancer, and cyclooxygenase (COX) and lipoxygenase (LOX) products contribute to the progression of the disease via promoting cell proliferation, motility, invasion, and angiogenesis (1–4). The underlying mechanism leading to eicosanoid pathway activation remains to be elucidated.

Phospholipase A_2 (PLA_2), catalyzes the hydrolysis of membrane glycerophospholipids, leading to production of free fatty acids and lysophospholipids. If the esterified fatty acid is arachidonic acid, this is converted in the prostate to prostaglandins by COX or hydroxyeicosatetraenoic acids by LOX. PLA_2 is a growing family of proteins, and in human, there are 10 secreted PLA_2 forms ($sPLA_2$), at least three isoforms of cytosolic PLA_2 ($cPLA_2$), and multiple isoforms of calcium-independent intracellular PLA_2 (5–9).

The aim of this study was, first, to determine the expression levels of $sPLA_2$ and $cPLA_2$ in patients with prostate cancer treated with androgen ablation therapy; second, to determine oncogenic potential of $sPLA_2$ and $cPLA_2$; and last, to determine the effect of specific $sPLA_2$-IIA inhibitors on prostate cancer cell growth. We have now obtained evidence that $sPLA_2$-IIA is constitutively expressed in remaining prostate cancer cells after 3 months of androgen ablation therapy. The increased $sPLA_2$-IIA has an oncogenic action that requires the activity of $sPLA_2$-IIA and of $cPLA_2$-α. Our newly designed $sPLA_2$-IIA inhibitors can block basal and $sPLA_2$-IIA–induced prostate cancer cell proliferation *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents. $sPLA_2$-IIA was purified from conditioned media produced by a Chinese hamster ovary cell line (5A2) stably transfected with the human $sPLA_2$-IIA cDNA, as described previously (10), and quantified by enzyme-linked immunosorbent assay (11). $sPLA_2$-IIA contained <0.1 ng of endotoxin per milligram of protein (*Limulus* amebocyte lysate pyrochrome assay; Associates of Cape Cod, Falmouth, MA) and was enzymatically active in a [^{3}H]arachidonate-labeled *Escherichia coli* membrane assay (10). Construction by site-directed mutagenesis, expression, purification, and characterization of the $sPLA_2$-IIA mutant enzyme $H_{48}Q$ will be described elsewhere.[8] $H_{48}Q$ was quantified by enzyme-linked immunosorbent assay (11). The mutant protein had no detectable enzyme activity in our *E. coli* membrane assay, but an activity of 2 to 4% of wild-type was reported in other studies (12). Two cyclic peptide $sPLA_2$-IIA inhibitors were synthesized using *N*-(9-fluoroenyl)methoxycarbonyl solid phase chemistry (Auspep, Melbourne, Australia) as described previously (10). c(2Nap)LS(2Nap)R is at least 10-fold more potent in binding $sPLA_2$-IIA and inhibiting $sPLA_2$ enzyme activity than cFLSYR. Peptides for *in vivo* use were exchanged into acetate buffer before lyophilization. The $cPLA_2$-α inhibitor pyrrolidine-1 was synthesized as described previously (13). Polyclonal anti-$sPLA_2$-IIA (160502; Cayman Chemicals, Sydney, Australia) and anti-$cPLA_2$-α (SC-438; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were purchased. The monoclonal anti-$sPLA_2$-IIA antibody 4A1 was raised and purified (Bioquest, Ltd., Sydney, Australia) as described previously (11).

Immunohistochemistry. Prostate tissues were all fixed in buffered 10% formaldehyde solution for approximately 24 hours and paraffin-embedded. Immunohistochemistry was performed on tissue sections (5 μm) using an avidin-biotin complex method with diaminobenzidine as a chromogen as described previously (14). The sections were subjected to microwave epitope retrieval before staining. Immunostaining was considered positive and specific when the intensity of staining with the post-immune IgG exceeded that observed with the preimmune IgG (isotype control) and with no IgG (method control). Specific staining was graded as described previously (15). In brief, the staining intensity was graded as low, intermediate, and high. The percentage of cells with the highest intensity staining was stratified into three scores:

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Note: P. Sved and K. Scott contributed equally to this work.

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[8] K. J. Bryant, P. W. Lei, M. Bidgood, M. Taberner, A. Cunningham, K. F. Scott, manuscript in preparation.

1, 0 to 33%; 2, 34 to 66%; and 3, 67 to 100%. Zero represented no cells with high intensity staining.

Cell Lines and Culture. The LNCaP, DU145, and PC3 cell lines were purchased from American Type Culture Collection (Rockville, MD). All cell cultures were maintained in RPMI 1640 (Sigma-Aldrich, Sydney, Australia), supplemented with 5% fetal bovine serum (FBS; ICN Biomedical, Sydney, Australia) for LNCaP and 10% FBS for DU145 and PC3 at 37°C in a humidified environment of 5% CO2. For experiments with the addition of androgens, LNCaP cells were cultured in the absence or presence of methyltrienolone (R1881; New England Nuclear Life Science, Wilmington, DE) for 72 hours in phenol red-free RPMI containing 5% charcoal-stripped FBS. 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.

Charcoal-Stripped Fetal Bovine Serum. Activated charcoal (40 g; 250–350 mesh; Sigma) was mixed with distilled water (1 L) and soaked for 1 hour at room temperature, and floating charcoal was removed. The soaking process was repeated until all floating charcoal was removed. Charcoal was then soaked in a minimal volume of acetone and allowed to dry by evaporation overnight in a fume hood. Dried charcoal was suspended in FBS to 0.4 mg/mL, incubated at 55°C for 10 minutes, and centrifuged for 5 minutes at 2000 $\times$ *g*; and the supernatant was filtered through a 0.2-μm filter. Because FBS has a low concentration of sex steroids, which makes it difficult to determine the efficiency of charcoal stripping, pooled human serum was used for validation of our protocol for charcoal stripping. Relative to unstripped serum, the concentration of testosterone, estradiol, and progesterone in charcoal-stripped serum was under the detection limit by competitive immunoassay (IMMULITE 2000; DPC, Los Angeles, CA), whereas the concentration of luteinizing hormone and follicular stimulating hormone, representatives of larger M_r proteins, were unchanged by microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL).

Reverse Transcription-Polymerase Chain Reaction. The effect of added androgen on the steady-state level of $sPLA_2$-IIA mRNA in LNCaP cells was measured by quantitative real-time reverse transcription-PCR. After cell treatments, total RNA was isolated using Trizol reagent. The first-strand cDNA was synthesized from 2 μg of total RNA using a combination of random hexamers and oligo(dT) as described previously (16). Primers were designed based on the human $sPLA_2$-IIA mRNA (NM_000300). Forward, 5′-TTTGTCACCCAAGAACTCTTAC-3′. Reverse, 5′-GGGAGGGAGGGTATGAGA-3′. Hypoxanthine phosphoribosyltransferase was used as the house-keeping gene, and its primer sequences were published previously (14). The PCR reaction (15 μL) contained 5.5 μL of water, 7.5 μL of 2$\times$ platinum quantitative PCR superMix-UDG (Invitrogen, Melbourne, Australia), 0.25 μL each of 0.01 mmol/L primers, 0.5 μL of 25$\times$ SYBR Green 1 dye (Molecular Probe, Sydney, Australia), and 1 μL of cDNA. The following protocol was used on ABI Prism 7000 sequence detection system: 50°C for 2 minutes, 95°C for 2 minutes followed by 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. After verification of parallelism in amplification efficiency (<10% variation in slopes on plots of crossing point cycles *versus* cDNA concentration) between $sPLA_2$-IIA and hypoxanthine phosphoribosyltransferase, the Δ-Δ method was used to calculate relative changes in mRNA levels of $sPLA_2$-IIA corrected for hypoxanthine phosphoribosyltransferase (HPRT) (17). For endogenous $sPLA_2$-IIA mRNA level, total RNA from LNCaP, DU145, and PC3 cells was isolated and reverse transcribed as above. Conventional end-time PCR was performed at an annealing temperature of 55°C.

Cell Proliferation Assay. Cells were plated at 1×10^4 per well in 96-well plates with 0.1 mL of media. Treatments were performed on cells at 70 to 80% confluence, in medium containing 5% FBS for 72 hours. After treatment, the number of viable cells was determined using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Sydney, Australia). In brief, 20 μL of solution were added to each well and incubated for 1 hour. The absorbance at 490 nm was measured with a microplate reader (Multiscan EX; Labsystems, Helsinki, Finland). Each experiment was performed in quadruplicate and repeated at least three times.

Flow Cytometric Analysis. LNCaP cells were seeded in 50-mL flasks and grown to 70 to 80% confluence before treatment for 72 hours. After trypsinization and cell counting, the treated or untreated cells (1×10^6) were suspended in PBS (1 mL) and incubated after the addition of 200 μL of Triton X-100 (0.4% stock) for 5 minutes at room temperature in the presence of 50 μL of propidium iodide solution (1 mg/mL stock) and 20 μL of RNase A Type I-A (50 mg/mL, stock; Sigma-Aldrich). DNA content per cell was measured by flow cytometry using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Analysis was performed on 10,000 events per sample.

PC3 Xenograft Study in Nude Mice. Animal studies were performed according to the guidelines for the ethical use of animals published by the National Health and Medical Research Council of Australia and with the approval of the University of New South Wales Animal Ethics Committee. Four- to 6-week-old athymic male nude mice (BALB/c) were inoculated subcutaneously with 1×10^7 PC3 cells. The cells were suspended in 0.1 mL of Dulbecco's modified Eagle's medium with 10% fetal bovine serum before injection and injected via a 26-gauge needle. When the size of the xenograft reached 5 $\times$ 5 mm (tumor volume, 65.5 mm^3 calculated as $V = \pi/6(d1.d2)^{3/2}$; ref. 18), mice were randomly assigned into four groups ($n = 15$ per group), *i.e.*, cFLSYR (1 mg/kg), cFLSYR (10 mg/kg), 2-naphthylalanine [c(2NapA)LS(2NapA)R; 1 mg/kg], and saline. cFLSYR was dissolved directly in sterile saline, whereas c(2NapA)LS(2NapA)R was diluted into sterile saline from a stock solution (40 mg/mL in dimethylformamide) immediately before use. The saline group received sterile saline containing 0.75% dimethylformamide. Subcutaneous injections were given three times weekly on the opposite flank to the xenograft site, and treatments were blinded to experimentalists throughout the study. Tumor growth was normalized for differing starting sizes by calculating relative tumor volume, *i.e.*, the ratio of each tumor volume on a given day to its volume on day 1 of treatment. The mice were treated for a period of 8 weeks unless culled due to tumor volume reaching 15 $\times$ 15 mm (1768 mm^3).

Statistical Analysis. The Number Cruncher Statistical System (NCSS, Kaysville, UT) was used for statistical analysis. Data were analyzed by χ^2 or analysis of variance (ANOVA) as appropriate. A two-tailed P value <0.05 was considered significant.

RESULTS AND DISCUSSION

Secreted Phospholipase A_2-IIA Expression Is Constitutively Activated in the Prostate Cancer Cells Remaining after Androgen Ablation Therapy. We searched the database of Serial Analysis of Gene Expression (SAGE) to determine the number of transcripts coding for individual PLA_2 enzymes in prostate cancer. The cDNA libraries used for SAGE were PR317 normal prostate and PR317 prostate cancer, respectively,[9] because both are derived from microdissected prostate tissues. We found that $sPLA_2$-IIA mRNA was 22 times higher in prostate cancer than normal prostate, whereas other members of the $sPLA_2$ (-IB, -IID, -IIE, -IIF, -III, -V, -X, -XII, and -XIII, also known as XIIB), $cPLA_2$ (-α, -β, and -γ), intracellular PLA_2 (-A), and platelet-activating factor acetylhydrolase families were either unchanged or not present. There are three published reports describing an increased $sPLA_2$-IIA mRNA (19) and protein levels (20, 21) in prostate cancer tissue and the enhanced $sPLA_2$-IIA expression was inversely related to 5-year patient survival (21). The chromosomal location of $sPLA_2$-IIA (1p35-ter) was found to overlap with a prostate cancer susceptibility locus CAPB (22). To verify the SAGE results and to extend them to cancer cells in the absence of androgens, we examined, immunohistochemically, $sPLA_2$-IIA expression in prostate cancer tissues from patients treated with androgen-ablation therapy for 3 months (100 mg of cyproterone acetate, twice daily) before radical prostatectomy. An undetectable serum prostate-specific antigen was confirmed in each patient before radical prostatectomy. Although the remaining cancer cells in these specimens are regarded qualitatively as being androgen independent, it is likely that the degree of androgen independence varies among these cells. Two antibodies were used for immunohistochemistry, and both showed the same staining pattern on consecutive sections. In the presence of androgens ($n = 50$), there was patchy cytoplasmic staining in benign glands (Fig.

[9] Internet address: www.ncbi.nlm.nih.gov/SAGE.

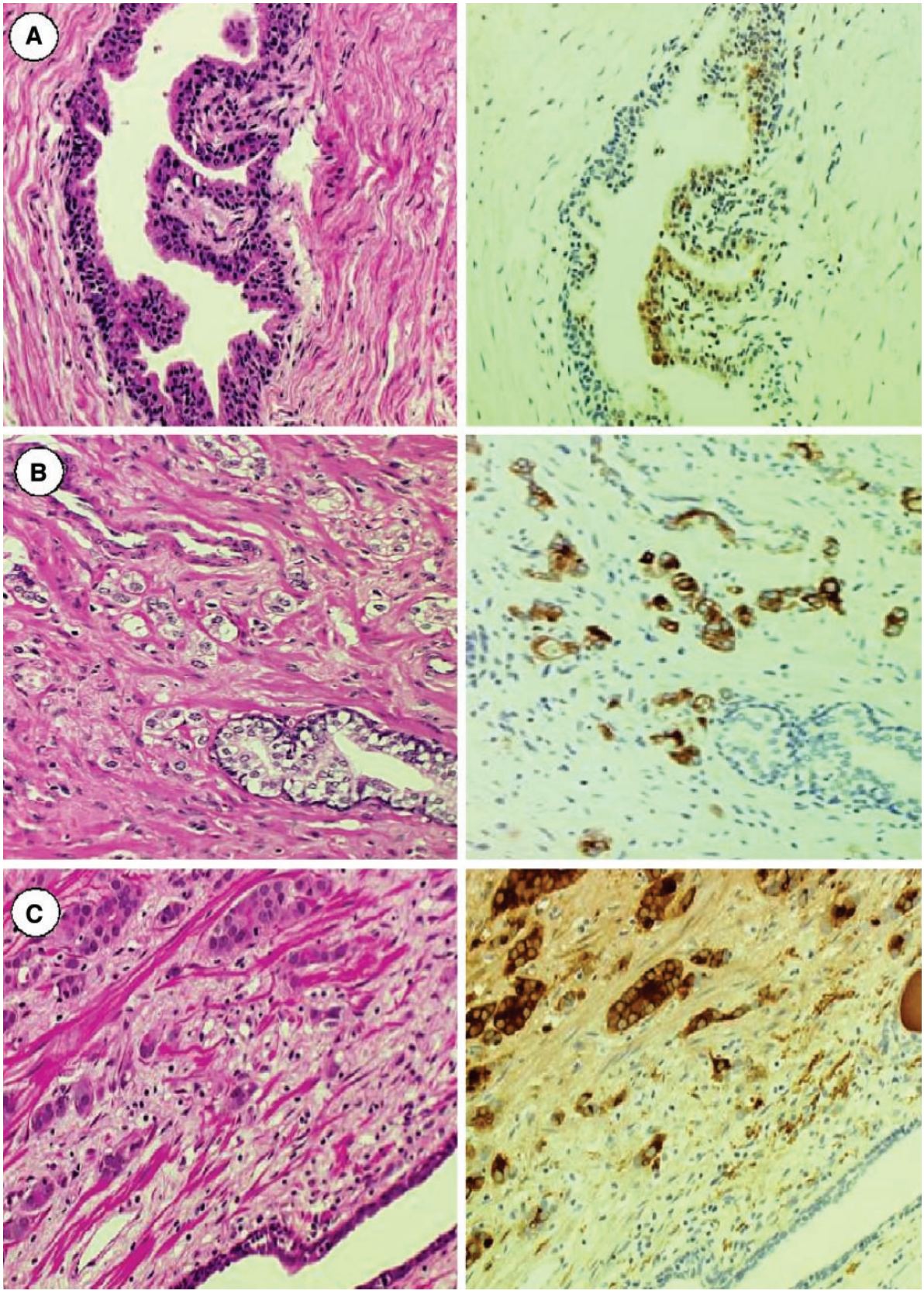

Fig. 1. Immunohistochemical analysis of $sPLA_2$-IIA in prostate tissues. In each *panel*, the *left section* is stained with hematoxylin and eosin, and the *right section* is stained with polyclonal anti-$sPLA_2$-IIA antibody (Cayman Chemicals). *A*, tissue sections from a patient without androgen-ablation therapy showing cytoplasmic brown staining in normal glands. *B*, sections from a patient without androgen-ablation therapy showing cytoplasmic brown staining in cancer cells. *C*, sections from a patient after androgen ablation therapy showing both normal (no staining) and neoplastic (brown staining) tissue. Magnification, ×40.

1*A*) adjacent to cancer cells and extensive cytoplasmic staining in cancer cells (Fig. 1*B*). With androgen ablation therapy ($n = 26$), benign glands showed substantially decreased staining, whereas cancer cells maintained $sPLA_2$-IIA expression (Fig. 1*C*). The percentage of stained cancer and of adjacent benign glands was then stratified into three scores (Fig. 2). No difference was found in immunohistochemical staining of $cPLA_2$-α between normal and cancer cells irrespective of androgen status (data not shown).

The lack of $sPLA_2$-IIA expression in benign glands after androgen deprivation (Fig. 2) suggests that expression of *$sPLA_2$-IIA* gene requires androgens. To verify that, we first searched the 5′-flanking region of the *$sPLA_2$-IIA* gene using MatInspector Release 5.3 (Genomatix, München, Germany) and found an androgen response element GAGGTAAATGGTATTCTC from −546 to −527. Second, we treated the androgen-responsive cell line, LNCaP, with metabolically stable synthetic androgen (methyltrienolone; R1881; 0.01–10 nmol/L) for 4, 8, and 24 hours and measured $sPLA_2$-IIA mRNA by real-time PCR. Indeed, there was a significant increase in the steady-state mRNA level of $sPLA_2$-IIA at 8 hours (Fig. 3). However, we could not find an androgen response element within 3 kb of the 5-flanking region of the $cPLA_2$ genomic DNA. Androgen treatment had no effect on $cPLA_2$ mRNA by real-time PCR (data not shown). Together, these findings suggest that expression of $sPLA_2$-IIA but not $cPLA_2$ is likely to be dependent on androgens in normal prostate. In cancer cells remaining after androgen ablation therapy, $sPLA_2$-IIA expression is constitutively active via an as yet unknown mechanism.

Oncogenic Action of Secreted Phospholipase A_2-IIA in Prostate Cancer Cells. To examine the biological relevance of constitutively expressed $sPLA_2$-IIA to cell growth, we treated LNCaP cells with various doses of human recombinant $sPLA_2$-IIA purified by immunoaffinity chromatography and monitored cell growth. We observed a consistent and dose-dependent stimulatory effect of $sPLA_2$-IIA on LNCaP cell growth as measured by the cell proliferation assay at doses as low as 1 nmol/L (Fig. 4*A*). This potent effect was blocked by the $sPLA_2$-IIA inhibitor, cFLSYR, at a 1:1 molar ratio of inhibitor to added enzyme (Fig. 4*B*).

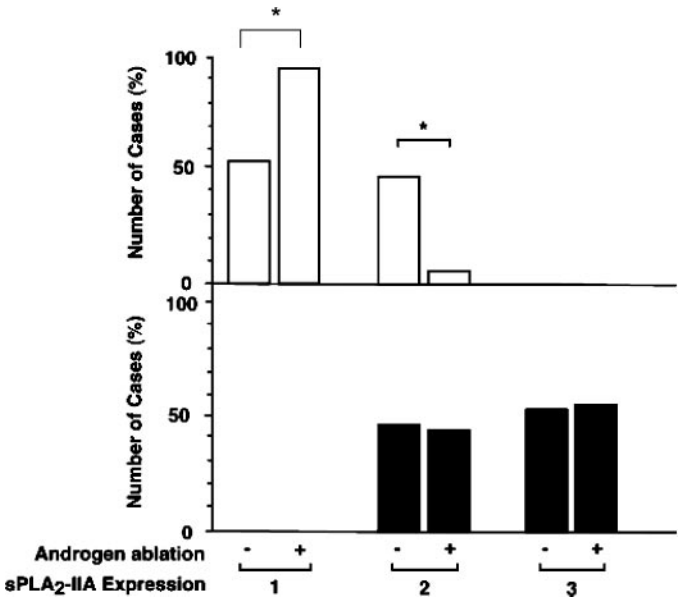

Fig. 2. Summary of immunohistochemical analysis. The extent of $sPLA_2$-IIA staining in benign glands (□) or cancer cells (■) from patients with (+, $n = 26$) or without (−, $n = 50$) androgen ablation therapy was graded on a three-point score, (1, 0–33% cells positive; 2, 33–66% cells positive; and 3, > 66% cells positive). Data are presented as the percentage of cases at each score. *, $P < 0.05$ *versus* benign glands without androgen ablation therapy by χ^2 analysis.

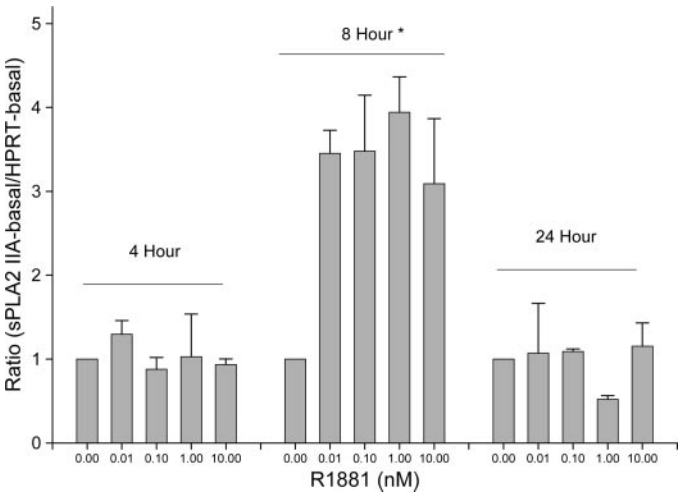

Fig. 3. Treatment of LNCaP cells with androgen-induced steady-state mRNA level of $sPLA_2$-IIA. LNCaP cells were grown in phenol red-free RPMI with 5% charcoal-stripped FBS and treated for 72 hours in media containing zero or increasing concentrations of R1881. mRNA levels of $sPLA_2$-IIA in treated cells relative to untreated control cells were determined by real-time reverse transcription-PCR. The data are shown as mean ± SD of triplicate determinations normalized to 100% for untreated cells and are representative of two independent experiments. *, $P < 0.001$ *versus* untreated cells by one-way ANOVA.

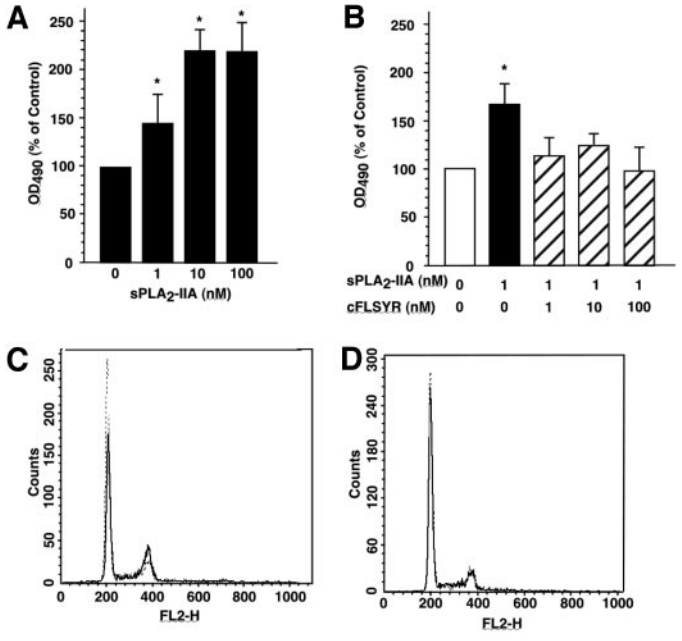

Fig. 4. Effect of addition of $sPLA_2$-IIA on prostate cell growth. LNCaP cells were grown in RPMI with 5% FBS and treated for 72 hours in media containing increasing concentrations of $sPLA_2$-IIA alone (*A*; ■) or a fixed concentration of $sPLA_2$-IIA and increasing concentrations of the $sPLA_2$-IIA inhibitor cFLSYR (*B*). Cell number relative to untreated control cells was determined by cell proliferation assay. Data are mean ± SD of quadruplicate determinations normalized to 100% for untreated cells. A_{490} of unstimulated cells was 0.29 ± 0.06 in *A* and 0.24 ± 0.03 for *B*. *, $P < 0.05$ *versus* untreated control by one-way ANOVA. Data are representative of three separate experiments. *C*, flow cytometric analysis of DNA content. LNCaP cells were grown as above and stimulated for 72 hours in the absence (*dotted lines*) and presence (*solid lines*) of the $sPLA_2$-IIA (100 nmol/L). *D*, same as *C* except that the *solid lines* represent the treatment of both $sPLA_2$-IIA (100 nmol/L) and $sPLA_2$-IIA inhibitor cFLSYR (100 nmol/L).

We next used flow cytometric analysis to determine how $sPLA_2$-IIA affects the distribution of LNCaP cells in different phases of the cell cycle. In $sPLA_2$-IIA–containing medium, the proportion of LNCaP cells in the G_1 phase decreased from 74 to 62% with a corresponding increase of cells in G_2-M phase in comparison with untreated cells (Fig. 4*C*). In the presence of both $sPLA_2$ and its inhibitor

(cFLSYR), the proportion of cells in G_1 and G_2 phase returned to basal levels (Fig. 4*D*). These results suggest the biological importance of the highly expressed $sPLA_2$-IIA in prostate cancer and demonstrate that the $sPLA_2$-IIA–induced cell proliferation can be attributed at least partly to an increased proportion of cells entering the G_2-M phase from the G_1 phase.

Oncogenic Action of Exogenous Secreted Phospholipase A_2-IIA Requires the Activity of Both Secreted Phospholipase A_2-IIA and Cytosolic Phospholipase A_2-α. In contrast to the essential *in vivo* role of $cPLA_2$-α in eicosanoid production under most physiologic conditions and in response to inflammatory stimuli, whether and if so, how $sPLA_2$-IIA increases eicosanoid production *in vivo* is unclear. Currently, two models are proposed to explain the action of $sPLA_2$ on eicosanoid production in cellular systems (23). In the first model, $sPLA_2$ binds directly to membrane phospholipids, and the released arachidonic acid serves as a substrate to produce eicosanoids. Lysophospholipids mediate indirect activation of endogenous $cPLA_2$-α via mobilization of calcium. The enhanced $cPLA_2$-α activity can in turn cause an increased production of eicosanoids. The second model is indirect modulation of intracellular eicosanoid pathways via cell surface glycosyl phosphatidyl inositol (GPI)-linked heparan sulfate proteoglycan receptors. The internalized $sPLA_2$ supplies arachidonic acid to downstream enzymes either directly or indirectly via activation of $cPLA_2$-α through mitogen-activated protein kinase–mediated phosphorylation. Enzyme activity of $sPLA_2$ is not obligatory for activation of the mitogen-activated protein kinase pathway.

To determine whether $sPLA_2$-IIA–induced prostate cancer cell proliferation depends on its enzyme activity, $H_{48}Q$ purified by immunoaffinity chromatography was used to treat LNCaP cells. Over the same dose range as the wild-type $sPLA_2$-IIA, $H_{48}Q$, which has no more than 2 to 4% of wild-type activity (12), had no growth-promoting effect (Fig. 5*A*), demonstrating that $sPLA_2$-IIA enzyme activity and thus its products are required for the proliferative effect of $sPLA_2$-IIA.

To evaluate whether $cPLA_2$-α mediates exogenously added $sPLA_2$-IIA action, we treated LNCaP cells with various doses of the selective $cPLA_2$-α inhibitor pyrrolidine-1 (13) with or without a constant effective dose of $sPLA_2$-IIA (1 nmol/L). Blockade of $cPLA_2$ abolishes $sPLA_2$-induced cell growth completely, demonstrating that $cPLA_2$-α activity is necessary for $sPLA_2$-IIA–dependent prostate cancer cell proliferation (Fig. 5*B*). Furthermore, the dose response at sub-micromolar to low micromolar range of pyrrolidine-1 is consistent with the dose required to block arachidonate release in a number of $cPLA_2$-α-expressing cells (13). Considering the common loss of annexins 1 (24) and 2 (14) in prostate cancer and the known inhibitory effect of the two annexins on $cPLA_2$-α activity, the activity of $cPLA_2$-α could be increased significantly in prostate cancer cells.

Cyclic Peptide Inhibitors of Secreted Phospholipase A_2-IIA Suppress Endogenous Secreted Phospholipase A_2-IIA–Dependent Proliferation *In vitro* and *In vivo*. Based on our finding that the growth-promoting $sPLA_2$-IIA is constitutively expressed in prostate cancer cells and the knowledge that production of arachidonic acid and lysophospholipids by PLA_2 is, in most cases, the rate-limiting step in eicosanoid synthesis, we have considered the potential of $sPLA_2$-IIA as a target for treatment of prostate cancer. We reason that a better outcome can be achieved with the PLA_2 inhibitor than with a COX inhibitor alone because the latter suppresses the production of prostaglandins only. The first $sPLA_2$-IIA inhibitor, LY311727, was rationally designed by Eli Lilly. Studies examining structure-activity relationships show that the Lilly inhibitor is relatively nonselective (25). We have previously shown that human $sPLA_2$-IIA is dose-dependently inhibited by a pentapeptide sequence comprising residues 70 to 74 of the native $sPLA_2$-IIA protein (^{70}FLSYK^{74}; ref. 26). Because of the inherent flexibility of the linear peptide sequence,

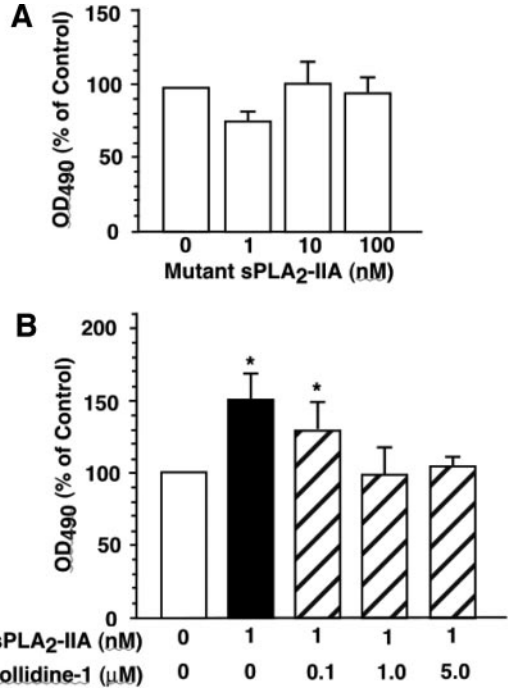

Fig. 5. Effect of inactive $sPLA_2$-IIA and inhibition of $cPLA_2$-α on $sPLA_2$-IIA–dependent proliferation. *A*, LNCaP cells were grown in RPMI and stimulated for 72 hours in media containing increasing concentrations of the inactive $sPLA_2$-IIA mutant $H_{48}Q$. *B*, $sPLA_2$-IIA (1 nmol/L) in the presence (▨) and absence (■) of increasing concentrations of the $cPLA_2$-α selective inhibitor pyrrolidine-1. Cell number was determined by cell proliferation assay. Data are mean ± SD of quadruplicate determinations expressed as percentages relative to untreated control cells (100%). A_{490} of unstimulated cells was 0.24 ± 0.03 in *A* and 0.33 ± 0.03 in *B*. In the presence of pyrrolidine-1 (5 μmol/L) alone, A_{490} was 0.38 ± 0.04. *, $P < 0.05$ *versus* untreated control by one-way ANOVA.

inhibition was weak in *in vitro* activity assays. We have recently designed two novel cyclic peptides (10), cFLSYR and a cyclic peptide in which F and Y are substituted with c(2NapA)LS(2NapA)R. Both have shown significant improvement in potency over linear peptides in $sPLA_2$-IIA inhibition assays and do not bind to a structurally related enzyme $sPLA_2$-IB.

To test the effect of blocking endogenous $sPLA_2$-IIA on cell growth, we first determined the basal mRNA levels of $sPLA_2$-IIA in three human prostate cancer cell lines. mRNA encoding $sPLA_2$-IIA was undetectable in DU145 compared with LNCaP and PC3 cells (Fig. 6*A*). We then tested the effect of individual inhibitors cFLSYR and c(2Nap)LS(2Nap)R on cell growth over a range of doses (1–100 nmol/L). LNCaP and PC-3 cell numbers were significantly decreased in the presence of inhibitor, and the smallest effective dose was 1 nmol/L (Fig. 6*B*). In contrast, neither of the inhibitors had an effect on DU145, presumably due to the lack of endogenous $sPLA_2$-IIA.

It is interesting to note the difference in response of LNCaP cells to the $sPLA_2$ inhibitors between Figs. 4 and 6. In the presence of exogenous $sPLA_2$-IIA in Fig. 4, cFLSYR did not reduce cell number below that of untreated cells at concentrations up to 100 nmol/L. However, in Fig. 6, c(2Nap)LS(2Nap)R at 1 nmol/L results in ~40% fewer cells than in control. Recent studies have shown that the effects of exogenous $sPLA_2$-IIA are mechanistically separable from those of endogenous intracellular $sPLA_2$-IIA in model cell systems (27). It is thus possible that $sPLA_2$-IIA may modulate cell growth by separate mechanisms depending on its cellular location. Also, the difference in potency between the two inhibitors (see Materials and Methods) may also contribute to the observation. Additional work will examine the detailed mechanism underlying the effect of both inhibitors in the presence or absence of exogenous $sPLA_2$-IIA.

In vivo, cFLSYR, at either 1 mg/kg or 10 mg/kg, and

c2(NapA)LS2(NapA)R at 1 mg/kg were injected three times a week subcutaneously to the flank opposite to the site of the PC3 xenograft. The treatment commenced only when the xenograft has reached the size of 5 × 5 mm. We found that inhibitor c(2NapA)LS(2NapA)R at 1 mg/kg and inhibitor cFLSYR at 10 mg/kg slowed the rate of growth of tumors from 5 weeks post-treatment commencement resulting in significant suppression of PC3 xenograft volume by 8 weeks of treatment compared with saline-treated mice (Fig. 7). cFLSYR had no effect at 1 mg/kg (data not shown). This *in vivo* order of potency of the two peptides correlates well with their relative potencies in *in vitro* enzyme activity assays (10). There were no visible side effects of either treatment including eating, drinking, and general behavior and no evidence of toxicity at the injection site. Although the inhibitory effect of both peptides is clear at 8 weeks after treatment-commencement, it is worth noting that, because pharmacokinetic data on these peptide inhibitors are not available at present, additional efficacy studies with optimized delivery route, dose, and dosing regimen are needed. It is nonetheless remarkable that blockade of xenograft-derived $sPLA_2$-IIA alone, as host-derived $sPLA_2$-IIA expression is largely confined to secretory cells in the gut in BALB/c mice and does not respond to agonist stimulation (28), seems to be sufficient to slow xenograft growth. In addition, evaluation of this effect relative to a COX-2–selective inhibitor would be valuable to corroborate the hypothesis that suppression of phospholipase A_2 is more beneficial than suppression of COX alone.

In summary, the normally androgen-responsive *$sPLA_2$-IIA* gene seems constitutively expressed in prostate cancer cells remaining after androgen ablation therapy. Exogenously added $sPLA_2$-IIA promotes prostate cancer cell proliferation by a mechanism that is dependent on the activity of both $sPLA_2$-IIA and $cPLA_2$-α. The loss of Annexin 1 and 2 in prostate cancer could enhance $sPLA_2$-IIA action by increasing the activity of $cPLA_2$-α. A better therapeutic outcome might be achieved through the use of PLA_2 inhibitors in the treatment of prostate cancer, particularly in the hormone refractory form.

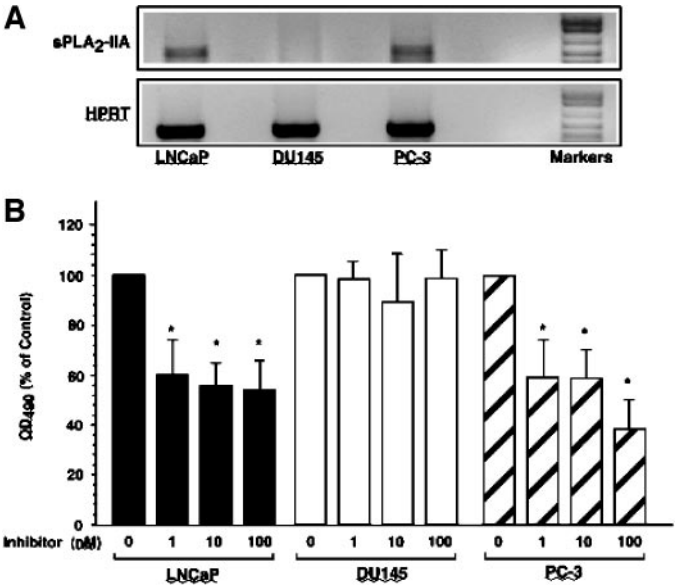

Fig. 6. Effect of $sPLA_2$-IIA inhibition on unstimulated prostate cancer cells. *A*, The endogenous expression of $sPLA_2$-IIA mRNA was evaluated by reverse transcription-PCR in three unstimulated prostate cancer cell lines (*LNCaP*, *DU145*, and *PC3*) grown in RPMI. Hypoxanthine phosphoribosyltransferase (HPRT) was used to as a positive control for RNA integrity and loading. *B*, Cells were then treated for 72 hours in the presence and absence of the $sPLA_2$-IIA inhibitor c(2Nap)LS(2Nap)R, and cell number was determined by cell proliferation assay. Data are mean ± SD of quadruplicate determinations expressed as percentages relative to untreated control cells (100%). LNCaP (■), A_{490} untreated cells 0.50 ± 0.03; DU145 (□), A_{490} untreated cells 0.73 ± 0.05; PC3 (▨), A_{490} untreated cells, 0.66 ± 0.01. ∗, $P < 0.05$ *versus* untreated control by one-way ANOVA.

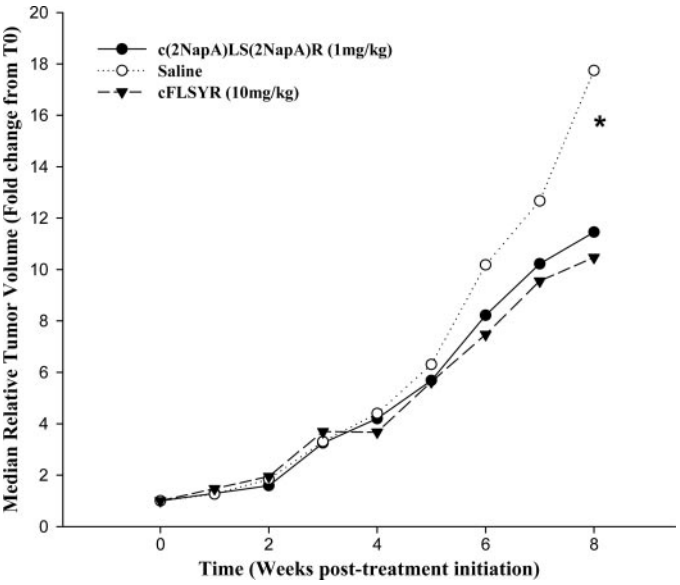

Fig. 7. Effect of $sPLA_2$-IIA inhibition on PC3 xenograft volume in nude male mice. Four- to 6-week-old athymic male nude mice were inoculated subcutaneously with 1×10^7 PC3 cells. Mice ($n = 15$ per group) that received injections of either of the two inhibitors showed a slower rate of growth from 5 weeks post-treatment commencement and had significantly smaller median relative tumor volume after 8 weeks of treatment compared with animals treated with saline. Median tumor volume at $T = 0$ for each group was as follows. Saline: median, 66.8 mm^3; range, 58.5–87.4 mm^3. cFLSYR (10 mg/kg): median, 69.8 mm^3; range, 57.9–105.1 mm^3. c(2NapA)LS(2NapA)R (1 mg/kg): median, 69.3 mm^3; range, 59.3–123.8 mm^3. Median tumor volumes for each group at 8 weeks were as follows. Saline: median, 1352.6 mm^3; range, 0–1998.1 mm^3. cFLSYR (10 mg/kg): median, 865.9 mm^3; range, 0–1991 mm^3. c(2NapA)LS(2NapA)R (1 mg/kg): median, 742.5 mm^3; range, 112.9–1945.6 mm^3. ∗, $P < 0.05$ for either treatment *versus* saline by repeated measures of two-way ANOVA (time × treatment) followed by multiple comparison test.

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