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# Regulation of Cox-2 by Cyclic AMP Response Element Binding Protein in Prostate Cancer: Potential Role for Nexrutine

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## Abstract

**We recently showed that Nexrutine$^R$, a *Phellodendron amurense* bark extract, suppresses proliferation of prostate cancer cell lines and tumor development in the transgenic adenocarcinoma of mouse prostate (TRAMP) model. Our data also indicate that the antiproliferative effects of Nexrutine$^R$ are mediated in part by Akt and Cyclic AMP response element binding protein (CREB). Cyclooxygenase (Cox-2), a pro-inflammatory mediator, is a CREB target that induces prostaglandin $E_2$ ($PGE_2$) and suppresses apoptosis. Treatment of LNCaP cells with Nexrutine$^R$ reduced tumor necrosis factor $\alpha$-induced enzymatic as well as promoter activities of Cox-2. Nexrutine$^R$ also reduced the expression and promoter activity of Cox-2 in PC-3 cells that express high constitutive levels of Cox-2. Deletion analysis coupled with mutational analysis of the Cox-2 promoter identified CRE as being sufficient for mediating Nexrutine$^R$ response. Immunohistochemical analysis of human prostate tumors show increased expression of CREB and DNA binding activity in high-grade tumors (three-fold higher in human prostate tumors compared to normal prostate; $P$ = .01). We have identified CREB-mediated activation of Cox-2 as a potential signaling pathway in prostate cancer which can be blocked with a nontoxic, cost-effective dietary supplement like Nexrutine$^R$, demonstrating a prospective for development of Nexrutine$^R$ for prostate cancer management.**

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**Keywords:** Cox-2 promoter activity, prostate cancer, CREB, inflammation, $PGE_2$.

## Introduction

Numerous studies have indicated a strong correlation between levels of arachidonic acid metabolites and accumulation of prostaglandins in carcinogenesis [1–5]. Cyclooxygenase-2 (Cox-2) is a rate-limiting enzyme that converts arachidonic acid into prostaglandins, which are key inflammatory signaling molecules. Deregulation of signal transduction pathways by proinflammatory stimuli has been implicated in tumor promotion. Increased synthesis of prostaglandin $E_2$ ($PGE_2$; resulting from upregulation of Cox-2 expression) inhibits apoptosis, stimulates angiogenesis, and promotes metastasis and immunosuppression, all of which play critical roles in the development and progression of cancer [6–10]. Because of the potential relationship between inflammation and cancer, proinflammatory pathways offer candidates for target-based chemoprevention agents [11–16].

Cytokines, mitogenic factors, tumor promoters, and stress-inducing agents induce the expression of Cox-2 [17]. Cox-2 is overexpressed in various types of tumor cells, including prostate cells, and is associated with resistance to apoptosis [18–30]. In addition, inhibition of Cox-2 using Cox-2–specific inhibitors showed promising antiproliferative and apoptosis-promoting effects, both *in vitro* and *in vivo*, in preclinical animal models [31–38].

Epidemiological studies have found a decreased incidence of prostate cancer among men who use nonsteroidal anti-inflammatory drugs (NSAIDs) [39–41]. Daily intake of acetylsalicylic acid (Aspirin) has been shown to reduce prostate cancer risk by 39% [42]. Furthermore, the efficacy of celecoxib, a selective Cox-2 inhibitor, has been evaluated in prostate cancer patients following radiotherapy or radical prostatectomy. Eight of 12 patients receiving 200 mg of celecoxib (twice daily) showed a significant reduction in serum prostate-specific antigen (PSA) levels after 3 months of treatment [43]. Subsequent studies by Smith et al. [44] have reported that celecoxib use after radical prostatectomy decreased mean PSA velocity by 3.4% ($P$ = .02). In contrast, the placebo control group showed a 3% increase in mean PSA velocity. Increased expression of Cox-2 has been shown to correlate with disease relapse [45]. These

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epidemiological studies and clinical trials showing the prostate cancer–preventive activity of Cox-2 inhibitors are encouraging and warrant more detailed studies [46].

Recently, we have shown that *Phellodendron amurense* bark extract, namely Nexrutine (Next Pharmaceuticals, Irvine, CA), inhibited the growth of prostate tumors [47]. We have also shown that Nexrutine prevented the development of adenocarcinoma in the TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) model through downregulation of Akt-mediated activation of cyclic AMP response element binding protein (CREB) [48]. CREB is a transcription factor that regulates a wide variety of genes by binding to cyclic AMP response element (CRE) elements in the promoter region, including Cox-2 [49–51]. However, it is not known whether Nexrutine-induced biologic effects are mediated through transcriptional regulation of Cox-2. In the present study, we examined the regulation of Cox-2 by CREB in prostate cancer and evaluated the ability of Nexrutine to inhibit CREB-mediated transcriptional activation of Cox-2.

## Materials and Methods

### *Preparation of Nexrutine*

Nexrutine was provided by Next Pharmaceuticals. Stock solutions of Nexrutine were prepared by dissolving 10 mg of Nexrutine in 10 ml of DMSO (1 mg/ml). This was diluted in growth media to obtain different concentrations (1–10 μg/ml).

### *Prostate Cancer Cell Lines*

Human prostate cancer cell lines LNCaP and PC-3 were grown and maintained as described previously [52–54].

### *Transient Expression Assays*

Transient transfections were performed using a Lipofectin reagent (Invitrogen, Carlsbad, CA), in accordance with the manufacturer's recommendations. Briefly Cox-2 (−1452/+59) and Cox-2 (−327/59) reporter plasmids (1 μg/well) and pRL-TK plasmid (50 ng/well; Renilla luciferase for normalization) were incubated with the Lipofectin reagent for 30 minutes at room temperature. The DNA–Lipofectin mixture was then added to the cells and incubated for 48 hours. Forty-eight hours after transfection, the cells were treated with solvent control or 5 μg/ml Nexrutine for 6 hours. Where indicated, cells were also treated with tumor necrosis factor α (TNFα) (20 ng/ml) for 30 minutes. Following treatments, cell extracts were prepared and assayed for luciferase activity, as described earlier [54]. Renilla luciferase activity was used to normalize transfection efficiency. Results are expressed as the ratio of firefly luciferase to Renilla luciferase at equal amounts of protein.

### *Immunohistochemistry*

Sections from formalin-fixed paraffin-embedded tissue blocks of prostate were cut and stained with phosphorylated CREB (pCREB) and CREB (Cell Signaling Technology, Inc., Danvers, MA). The secondary and tertiary antibodies were biotinylated link and streptavidin horseradish peroxidase (Biocare 4 plus Kit; Biocare Medical, Concord, CA, or Vector Laboratories, Burlingame, CA).

### *Preparation of Extracts from Prostate Tumors and CREB DNA Binding Activity*

Whole-cell extracts from normal and high-grade prostate tumors ($n = 3$ each) were prepared using Active Motif nuclear extract preparation kit (Active Motif, Carlsbad, CA). CREB DNA binding activity was measured in normal human prostates and high-grade prostate tumors by using TransAM CREB (Active Motif). Briefly, the extracts were incubated with a CREB consensus oligonucleotide that was immobilized in a 96-well plate. A primary antibody specific for an epitope on the bound and active forms of CREB is then added, followed by subsequent incubation with secondary antibody and developing solution. Following this incubation with the developing solution, CREB activity was measured colorimetrically at 450 nm with a Spectramax plate reader (Molecular Devices, Sunnyvale, CA).

### *Determination of $PGE_2$ Levels*

$PGE_2$ levels were determined using $PEG_2$ Biotrak enzyme immunoassay system (RPN 222), in accordance with the manufacturer's recommendations (Amersham Biosciences Corp., Piscataway, NJ).

### *Reverse Transcriptase–Polymerase Chain Reaction for Cox-2*

RNA isolated from LNCaP and PC-3 cells was amplified using Cox-2 and β-actin primers, as described [55]. Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using Access RT-PCR system (Promega Corporation, Inc., Madison, WI).

### *Statistical Analysis*

Data are presented as average ± SD, and significance was determined using Student's *t* test. Differences between the experimental groups were considered significant at $P < .05$.

## Results and Discussion

### *Modulation of Cox-2 By Nexrutine*

We measured the expression of Cox-2 using RT-PCR and immunoblot analysis in androgen-responsive LNCaP and androgen-independent PC-3 cells. Figure 1 shows that PC-3 cells constitutively express high levels of Cox-2. In contrast, LNCaP cells express low levels of Cox-2 (data not shown). These data confirm published reports examining the expression of Cox-2 in prostate cancer cells [26,27]. We next investigated whether Nexrutine treatment modulates Cox-2 expression in PC-3 cells. As also shown in Figure 1, Nexrutine reduced the message levels of Cox-2 by about 10% within 30 minutes of treatment ($P = .01$) and maintained that level for 6 hours. Immunoblot analysis using whole-cell extracts also consistently showed undetectable to low levels

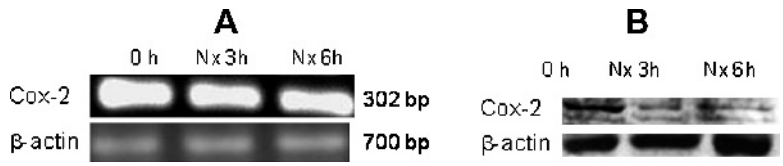


**Figure 1.** *Nexrutine treatment reduces the (A) expression of Cox-2 as determined by RT-PCR and Western blot analysis. (A) Total RNA was extracted from PC-3 cells treated with Nexrutine for 3 and 6 hours, as described in Materials and Methods. RNA was amplified using one-step RT-PCR. (B) Equal amounts of cell extracts were fractionated on a 10% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. The blotted membrane was blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 (blocking solution) and incubated with indicated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; Cell Signaling Technology, Inc.; Upstate Cell Signaling, Lake Placid, NY; Calbiochem, San Diego, CA), followed by incubation with horseradish peroxidase–conjugated anti-rabbit IgG antibody (Sigma, St. Louis, MO) in blocking solution. Bound antibody was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent Substrate, following the manufacturer's directions (Pierce, Rockford, IL). The blot shown is a representative blot of three independent experiments. All the blots were stripped and reprobed with β-actin to ensure an equal loading of protein.*

of Cox-2. As a reflection of Cox-2 activity, we also measured the levels of $PGE_2$ with and without Nexrutine and TNFα using ELISA (Amersham Biosciences Corp.). Androgen-responsive LNCaP cells were used in these experiments because endogenous levels of Cox-2 are low compared to PC-3 cells. Increased production of $PGE_2$ was observed in LNCaP cells treated with TNFα. Although 1 μg/ml Nexrutine reduced the production of $PGE_2$ ($P$ = .03; Figure 2), the decrease was highly significant with treatment at 10 μg/ml ($P$ = .007). As shown in Figure 2, the increase in the production of $PGE_2$ with treatment at 10 μg/ml, compared to 5 μg/ml, was not statistically significant ($P$ = .42). We had previously shown that Nexrutine promotes apoptosis in prostate cancer cells under the same experimental conditions [47].

*Nexrutine Reduces TNFα-Induced Cox-2 Promoter Activity*

Although Nexrutine reduced the expression of Cox-2 and Cox-2–mediated production of $PGE_2$, the mechanisms that regulate its expression are not known. Cox-2 has been shown to be regulated at multiple levels, including transcription, posttranscription, and protein turnover [56,57]. To investigate whether Nexrutine regulation of Cox-2 transcription occurs through the modulation of its promoter, the full-length Cox-2 promoter construct containing 5′-flanking sequences of the human *Cox-2* gene (−1432/+59) was produced and transfected into LNCaP and PC-3 cells. Forty-eight hours after transfection, the cells were treated with Nexrutine (5 μg/ml) for 6 hours. During the last 30 minutes of the 6-hour incubation period, the cells were coincubated with TNFα (20 ng/ml). Luciferase activity was measured as described in Materials and Methods. As shown in Figure 3, Cox-2 promoter activity was induced consistently in response to TNFα, and this TNFα-induced promoter activity was reduced to basal

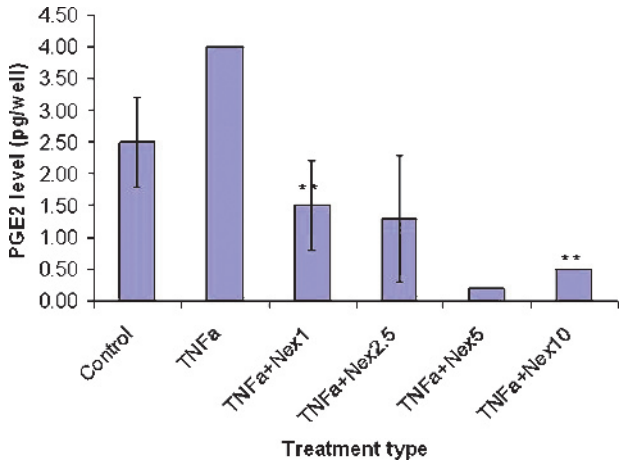


**Figure 2.** *Nexrutine treatment reduces TNFα-induced levels of $PGE_2$. LNCaP cells were set up in a 96-well plate at a density of 4000 cells/well. Seventy-two hours later, the cells were preincubated with media containing different concentrations of Nexrutine, in triplicate (0, 1, 2.5, 5, and 10 μg/ml), for 6 hours. The cells were then stimulated with TNFα (20 ng/ml) for 30 minutes, and $PGE_2$ levels were measured using an enzyme-linked immunoassay system (RPN 222) from Amersham Biosciences Corp., as per the manufacturer's recommendations. The data shown here are presented as the average ± SD of two independent experiments.*

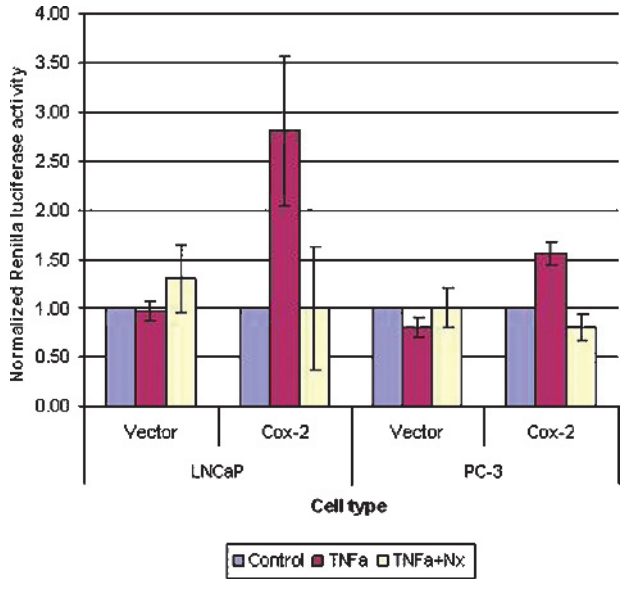


**Figure 3.** *Nexrutine reduces TNFα-induced Cox-2 promoter activity in LNCaP cells. Transient transfections were performed with −1452/+59 Cox-2 reporter plasmid (1 μg/well) and pRL-TK plasmid (50 ng/well; Renilla luciferase for normalization), as described in Materials and Methods, using Lipofectin reagent. Forty-eight hours after transfection, the cells were treated with Nexrutine (5 μg/ml) for 6 hours and stimulated with TNFα (20 ng/ml) for 30 minutes. Firefly and Renilla luciferase activities were measured in the extracts prepared from these using the Dual-Luciferase Reporter Assay System (Promega Corporation, Inc.), in duplicate samples containing equal amounts of protein. Renilla luciferase activity was used to normalize transfection efficiency. Results are expressed as the ratio of firefly luciferase to Renilla luciferase at equal amounts of protein. The data shown here are representative of four experiments conducted with two different preparations of plasmid.*

levels in Nexrutine-treated cells. As shown in Figure 3, LNCaP cells are more responsive to TNFα than are PC-3 cells. Vector-transfected (pGL3basic) cells showed no significant response to either TNFα or Nexrutine.

*Sequence Elements Necessary for Nexrutine-Mediated Reduction in Cox-2 Promoter Activity*

We used PC-3 cells in subsequent studies to examine Cox-2 regulation because they constitutively express high levels of Cox-2. PC-3 cells were transfected with −1452/+59 and −327/+59 human Cox-2 promoter constructs, as described in Materials and Methods. Following 48 hours of transfection, the cells were treated with Nexrutine (5 μg/ml) for 6 hours (time point based on reduction in Cox-2 expression), and luciferase activity was measured using the Dual-Luciferase assay (Materials and Methods). Nexrutine significantly reduced the promoter activity of deletion constructs −1452/+59 and −327/+59 ($P = .006$ and .006, respectively). Analysis of these results indicates that sequences between −327/+59 were sufficient to mediate Nexrutine-induced downregulation of Cox-2 promoter activity (Figure 4). This sequence contains potential binding sites for transcription factors NFκB (−223/−214), CRE (−59/−53), and NF-IL-6 (−132/−124). Based on previously published data that showed an important role for CREB in mediating Nexrutine-induced biologic effects, we tested whether the CRE site in the Cox-2 promoter plays an important role in Nexrutine-mediated downregulation of promoter activity [47,48].

*CRE Is Sufficient for Nexrutine-Mediated Reduction in Cox-2 Promoter Activity*

To examine the direct involvement of CRE in mediating Nexrutine-inhibited Cox-2 promoter activity, transient expression assays were performed with a reporter plasmid that had a mutation in the CRE site transfected into PC-3 cells. The promoter activity obtained with the mutated CRE binding site was about 60% of that with the wild-type CRE site (−327/+59). However, when the mutant construct was used in the transfections, Nexrutine marginally reduced promoter activity (about 10%, $P = .005$; Figure 4). These data indicate that the CRE site is critical for mediating Nexrutine-mediated inhibition of Cox-2 promoter activity. This is also consistent with our published data showing that Nexrutine reduces the levels of pCREB and CREB DNA binding activity in prostate cancer cells, as well as in prostate tumors from TRAMP mice [47,48]. Although this is the first report to show an important role for CREB in the transcriptional regulation of Cox-2 in PC-3 cells, these data are consistent with published reports demonstrating a role for CREB in the transcriptional regulation of Cox-2 in keratinocytes [58]. A potential role for NFκB or NF-IL-6, either directly or in association with CREB, cannot be ruled out at present because the Cox-2 promoter contains binding sites for these factors.

*Expression of CREB in Human Prostate Tumors*

Formalin-fixed paraffin-embedded samples of human prostate tissues were studied by immunohistochemistry to determine whether expression of CREB can serve as a marker for prostate cancer. Histologic sections of tissue arrays containing eight cases each of normal, low, and high Gleason grades from prostatectomy specimens were examined. The intensity of CREB and pCREB expression was scored semiquantitatively as 1+ (*no expression*), 2+ (*low expression*), 3+ (*moderate expression*), and 4+ (*high expression*), as described previously [48]. CREB and pCREB staining was observed mainly in the nucleus. Furthermore, CREB and pCREB staining was detected only in epithelial cells (benign or malignant), but not in stromal cells (Figure 5). Fifty percent of the high-Gleason-grade (≥ 8 of 10) tumors showed 4+ staining, whereas 75% of low-Gleason-grade (≤ 7 of 10) tumors showed 2+ staining. These data suggest that CREB is modulated during prostate cancer progression and that activation of CREB may drive prostate carcinogenesis through transcriptional regulation of genes such as *Cox-2* that are involved in inflammation. We also measured CREB binding activity in extracts of frozen normal human prostate

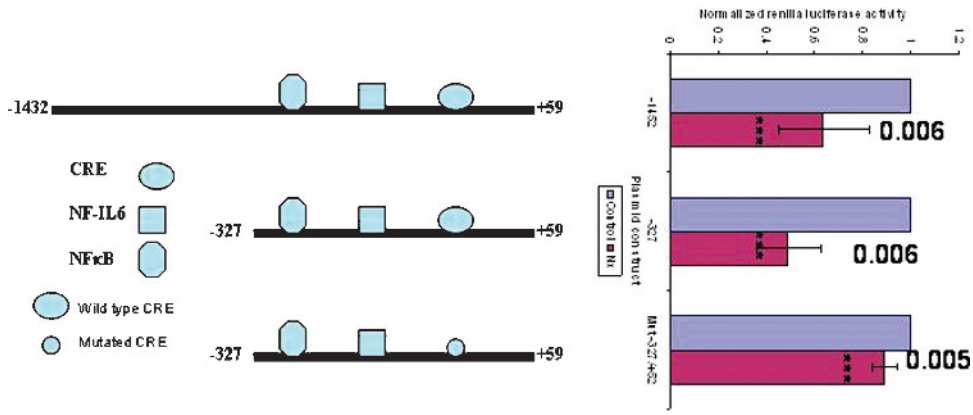


**Figure 4.** *Sequence elements between −327/+59 are sufficient for mediating Nexrutine response. Diagrammatic representation of Cox-2 promoter constructs used in transfection experiments. Transient transfections were performed as described above in Figure 3 using the indicated constructs of Cox-2 promoter, and promoter activity was measured following treatment with Nexrutine (5 μg/ml for 6 hours). The results are presented as the average ± SD of three independent experiments conducted in triplicate.*

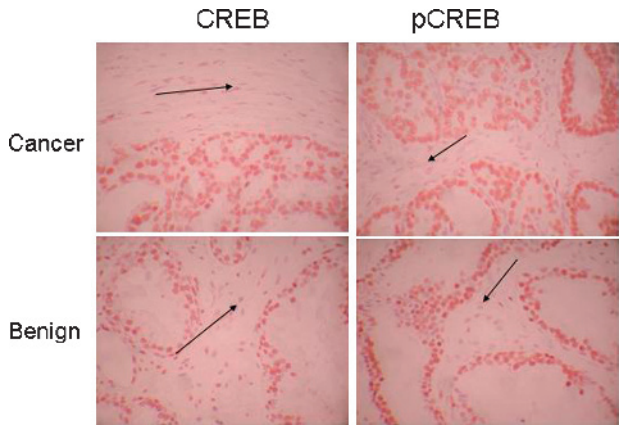


**Figure 5.** *Detection of CREB and pCREB in prostate tumor specimens by immunohistochemistry. A human prostate tissue array containing low-grade and high-grade tumors with paired normal prostate was stained with CREB and pCREB. CREB and pCREB were used at a dilution of 1:100 in PBS and incubated overnight at 4° C. Immune complexes were revealed using a universal secondary antibody (100 μl for 30 minutes) followed by chromogen, as described in Materials and Methods. Negative controls were included by omitting the primary antibody (data not shown).*

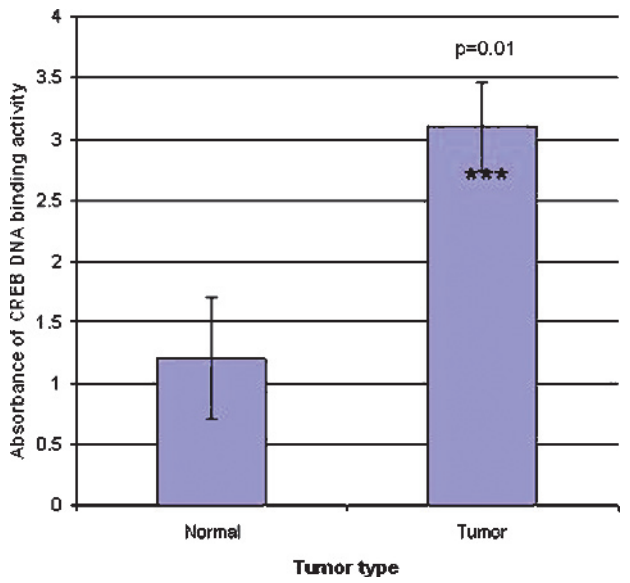


**Figure 6.** *CREB DNA binding activity in normal prostate and human prostate tumors. Briefly, the extracts prepared from normal and tumor tissues were incubated with a CREB consensus oligonucleotide that was immobilized in a 96-well plate. A primary antibody specific for an epitope on the bound and active forms of CREB is then added, followed by subsequent incubation with secondary antibody and developing solution. Following this incubation with the developing solution, CREB activity was measured colorimetrically at 450 nm.*

tissues and prostate tumors (three samples from each). As shown in Figure 6, CREB DNA binding activity was low in normal prostate tissues and increased by approximately three-fold in tumor samples ($P = .01$). These preliminary data from immunohistochemical studies and DNA binding activity suggest that CREB levels and transcriptional activation are modulated during prostate cancer evolution.

Chemoprevention is an important and practical strategy for the management of cancer. Many naturally occurring substances such as phytochemicals (compounds derived from plants such as fruits and vegetables) have been identified as potential chemopreventive agents. Furthermore, chronic inflammation has been shown to be associated with an increased risk of various malignancies, including prostate cancer [10–14]. Although the precise mechanism through which inflammation induces cancer is not clear, inflammation has been shown to contribute to the development of 15% of all cancers. Interestingly, hyperplastic changes that are closely associated with chronic inflammation have been identified in the prostate [59]. The terms *postatrophic hyperplasia* and *proliferative inflammatory atrophy* have been used to describe these morphologic changes [60]. Overexpression of Cox-2 has been observed in these lesions [61,62]. It has also been shown that prostate cancer is frequently accompanied by chronic inflammation [63] and that biochemical relapse following radical prostatectomy is more frequent in patients with high-grade inflammation [64]. These observations suggest that chronic inflammation and associated Cox-2 overexpression may be early events in the pathogenesis of "inflammation-related" prostate cancer and may be associated with a more aggressive phenotype. Identification of signaling pathways associated with chromic inflammation may lead to preventive strategies for the successful management of not only inflammation but also progression of prostate cancer. Activation of CREB in prostate epithelial cells may induce Cox-2 transcription, leading to inflammation that supports tumor growth through inhibition of apoptosis and increased angiogenesis. The results presented in this article show that reducing the levels and activity of CREB by Nexrutine may restore the sensitivity of prostate cells to apoptotic stimuli through downregulation of Cox-2. Reduction of CREB activity may be one targeted approach toward the management and/or prevention of prostate cancer. As discussed above, epidemiological studies have already shown that people who regularly take NSAIDs have a lower risk of developing cancer than people who do not take them. However, long-term use of NSAIDs has also been shown to be associated with gastrointestinal or cardiovascular side effects. The use of Nexrutine may reduce such risks because of its nontoxic nature. Furthermore, being a complex mixture, Nexrutine may target multiple targets, including CREB, NFκB, Cox-2, and cyclin D1, that play a critical role in the carcinogenesis process. The development of compounds, or a combination of compounds, targeting multiple targets has been proposed as an ideal approach for successful cancer management [65]. We have identified CREB-mediated activation of Cox-2 as a signaling pathway in prostate cancer that can be blocked with a nontoxic cost-effective dietary supplement such as Nexrutine, demonstrating a potential for the development of Nexrutine for prostate cancer management.

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