The Cyclooxygenase 2-specific Nonsteroidal Anti-inflammatory Drugs Celecoxib and Nimesulide Inhibit Androgen Receptor Activity via Induction of c-Jun in Prostate Cancer Cells

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Abstract
Nonsteroidal anti-inflammatory drugs (NSAIDs) play potential roles in cancer chemoprevention. In this study, we investigated the effects of NSAIDs on androgen receptor (AR)-mediated functions in prostate cancer cells. We found that two cyclooxygenase 2-specific NSAIDs, celecoxib and nimesulide, dramatically reduced the expression of androgen-inducible genes, such as prostate-specific antigen, hK2, and the FK506-binding protein 51 (FKBP51). We demonstrated that both NSAIDs repressed AR-mediated activation of prostate-specific antigen and hK2 promoter activity as well as AR protein expression. Finally, our findings suggested that overexpressed c-Jun by the NSAIDs not only inhibited the function of AR but also directly repressed AR expression at the transcription level. Our findings provide a strong rationale for celecoxib and nimesulide as potential agents for prostate cancer prevention and/or treatment.

Introduction
Epidemiological studies, laboratory bioassays, and human clinical intervention trials strongly support a protective role of NSAIDs against cancer development (1, 2). For example, several epidemiological studies showed a significant inverse association between the intake of aspirin and other NSAIDs and the risk of colorectal cancer in the general population (1, 2). NSAIDs are effective in reducing existing polyps in familial adenomatous polyposis patients and reduce the tumor burden in several animal models of colorectal cancer. A similar inverse association has been described for other cancers including cancer of the breast, ovary, rectum, and esophagus (1, 2). For prostate cancer, there are a few studies showing a weak (but not statistically significant) inverse association between NSAIDs and the cancer (3). However, a recent report by Nelson and Harris (4) indicated a 70% reduction in the risk of prostate cancer among NSAID users. A more recent, community population-based study (3) showed that there is a significant reduction in prostate cancer risk among men aged 60 years or older who were daily users of NSAIDs. Interestingly, the study found that the strongest inverse association with an 83% reduction in risk was among men of age 70 years or older. The authors attributed this to the highest use of NSAIDs in this group of men studied and suggested NSAIDS as potential chemopreventive agents for prostate cancer. However, the molecular mechanism(s) supporting this hypothesis remains to be elucidated.

The AR, a ligand-dependent transcription factor and a member of nuclear receptor superfamily, plays a central role in androgen action in the prostate (5, 6). Androgens and the AR have been strongly suggested as risk factors for prostate cancer development (5–7). Both epidemiological and biochemical studies strongly support androgen deprivation-based chemoprevention and chemotherapy for prostate neoplastic diseases (5–7). The AR could be one of the important targets for intervention at all stages of development and progression of prostate cancer.

We reported previously (8) that flufenamic acid could inhibit AR-regulated gene expression. In this study, we further describe our novel findings on the regulation of AR-mediated function by the two COX-2-specific NSAIDs, celecoxib and nimesulide. Our data suggested that NSAIDS strongly induced expression and phosphorylation of c-Jun, which inhibited the expression of AR-regulated genes as well as AR itself. Both mechanisms seem to contribute to their potent inhibitory effect on the function of the AR. Our study provides a potential molecular mechanism linking the down-regulation of AR-mediated function in prostate cancer cells by celecoxib and nimesulide to their anti-prostate cancer activity.

Materials and Methods
Cell Culture. Human prostate cancer cell lines LNCaP (American Type Culture Collection, Manassas, VA) and LAPC-4 (kindly provided by Dr. Charles L. Sawyer; Ref. 9) were maintained in RPMI 1640 (Mediatech, Hercules, CA) containing 5% FBS (Biofluids, Rockville, MD) at 37°C and 5% CO₂. To avoid potential interference of existing steroids in FBS, the media were first replaced by serum-free RPMI 1640 for 24 h. Cells were then cultured in RPMI 1640 with 5% charcoal-stripped FBS supplemented with or without 1 nM Mib (New England Nuclear, Boston, MA), a nonmetabolizable, synthetic androgen.
Celecoxib and Nimesulide Inhibit AR Activity

<table>
<thead>
<tr>
<th>NSAIDs</th>
<th>Selective inhibitor</th>
<th>LNCaP (IC₅₀)a</th>
<th>LAPC-4 (IC₅₀)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>COX-1 and -2</td>
<td>&gt;1000</td>
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</tr>
<tr>
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<td>COX-1 and -2</td>
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<td>Ketoprofen</td>
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<td>Flurbiprofen</td>
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<td>&gt;300</td>
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<tr>
<td>Nimesulide</td>
<td>COX-2</td>
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<td>104</td>
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<td>COX-1 and -2</td>
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<td>COX-2</td>
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<tr>
<td>Indomethacin</td>
<td>COX-1 and -2</td>
<td>&gt;300</td>
<td>170</td>
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</table>

a IC₅₀ is given in μM.
b A metabolite of sulindac loses NSAID activities.
c N/D, not determined.

Growth Response and PSA and hK2 Levels. Cells were plated in 24-well plates at 2 × 10⁵ cells/well. Forty-eight h after plating, cells were treated with celecoxib or nimesulide (LKT Lab, St. Paul, MN) and other NSAIDs as shown in Table 1 at different doses in the presence or absence of Mib. MTS assay, a non-radioactive cell proliferation assay, composed of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI) was performed to determine cell proliferation 6 days after the treatment. To measure secreted PSA and hK2 levels, 400 μL of spent medium from cells treated for 6 days were collected. PSA and hK2 proteins levels were determined using specific immunoassays (Mayo Immunochemical Core Facility). These measurements were used to the calculate 50% inhibitory concentration (IC₅₀) of each of the NSAIDs.

Western Blot Analysis. Cells were seeded at 1 × 10⁵ cells/plate in 100-mm dishes. Cells grown in log phase were cotreated with 1 mM Mib and different concentrations of celecoxib or nimesulide for 15 or 24 h. The cells were collected by centrifugation and washed with cold PBS. Cell lysates were prepared in radioimmunoprecipitation assay buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS plus freshly added protease inhibitors, 100 μg/mL phenylmethylsulfonyl fluoride, 30 μL/mL aprotinin, and 1 mM sodium orthovanadate) and used for Western blot analysis. The sample filters were immunoblotted with c-Jun, phospho-c-Jun (Cell Signaling, Beverly, MA), AR (PharMingen, San Diego, CA), FKBP51 (a gift from Dr. D. O. Toft; Mayo Clinic) specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

Transfections and Transcriptional Reporter Assays. LNCaP cells were plated into 60-mm dishes. Cells at 50–70% confluence were transfected with the appropriate constructs [6-kb PSA promoter-pGL3, AR promoter (~74/+87)-pGL3, AR promoter (~1380/+577)-pGL3, hK2 3xARE-SV40 minimal promoter-pGL3, or empty pGL3 vectors] by using the method described previously (10). Twenty-four h after transfection, cells were treated with celecoxib or nimesulide in combination with Mib or ethanol. Whole cell lysate was prepared for luciferase assay according to the manufacturer’s instructions (Promega). CMV-B-gal expression vector was also cotransfected for normalization of transfection efficiency. Each transfection was done three times, and SDs were calculated.

Statistics. The data were analyzed by Student’s t test. P < 0.05 was accepted as the level of significance.

Results

Celecoxib and Nimesulide Inhibited the Expression of Androgen Up-Regulated Genes. We were interested in finding out whether commonly used NSAIDs have the ability to regulate the function of the AR in prostate cells. Thus we studied the effects of several NSAIDs on inhibition of androgen action and growth in prostate cancer cells. Using PSA and hK2, two well-established AR target genes, as markers, we tested the effects of a panel of 11 NSAIDs on androgen action in two androgen-responsive human prostate cancer cell lines, LNCaP and LAPC-4, respectively. Among the NSAIDs tested, COX-2-specific inhibitors seem to have a higher potency than other NSAIDs in inhibiting androgen action. Celecoxib and nimesulide showed the lowest IC₅₀ concentrations in both cell lines (Table 1). Because of their highest potency on inhibition of cell growth and androgen function, celecoxib and nimesulide were chosen for additional studies. (Fig. 1) illustrates that expression of PSA and hK2 was suppressed by celecoxib and nimesulide in a dose-dependent manner in the two cell lines. Significant inhibitory activity was observed for celecoxib at a concentration of 10 μM for both PSA and hK2. Nimesulide at 10 μM resulted in a similar inhibition of PSA, although a higher concentration was required to achieve significant down-regulation in LAPC-4 cells. Recently, we discovered that FKBP51, an immunophilin, is up-regulated by androgens (11). Similarly, it was found that androgen-up-regulated FKBP51 protein expression was alleviated by celecoxib and nimesulide treatment as measured by Western analysis using specific antibody (Fig. 2A). These results suggest these NSAIDs are potent inhibitors of AR-induced gene expression.

Celecoxib and Nimesulide Inhibited AR-induced Gene Expression and AR Promoter Activity at the Transcription Level. To test whether celecoxib and nimesulide can directly repress the promoters of AR-dependent genes, we performed reporter assays using a PSA promoter-luciferase construct. As can be seen in Fig. 2B, celecoxib and nimesulide significantly reduced the androgen-induced PSA promoter activity at a concentration as low as 25 μM. Because the AR binds directly to the ARE of target genes for androgen action, we also tested another reporter construct containing three tandem repeats of ARE derived from the hK2 promoter (pGL3 hK2 3xARE-SV40
The result shown in Fig. 2B demonstrated that both celecoxib and nimesulide significantly reduced AR/ARE-mediated gene expression (P < 0.05). Thus, celecoxib and nimesulide acted as potent inhibitors of AR-mediated gene transcription.

To determine whether celecoxib and nimesulide may directly affect the transcriptional activity of the AR gene, transcriptional reporter assay was performed in LNCaP cells using a luciferase reporter plasmid containing the AR promoter (1380/577). Compared with control vector alone, cells transfected with the AR promoter revealed significantly higher luciferase activities, as expected (Fig. 3A). However, celecoxib and nimesulide, at the concentrations used, repressed the transcription activities of the promoter (Fig. 3A). Furthermore, Western analysis using AR-specific antibody indicated that AR protein expression was reduced by celecoxib and nimesulide (Fig. 2A) at concentrations used in the transfections. Taken together, these results suggest that celecoxib and nimesulide are potent inhibitors of AR function at least partially through down-regulation of AR expression.

Enhanced Expression and Phosphorylation of c-Jun by Celecoxib and Nimesulide in LNCaP Cells. To further dissect the molecular mechanisms underlying NSAID-mediated inhibition of AR function, we next examined the expression of c-Jun in celecoxib- and nimesulide-treated LNCaP cells by Western blot analysis. We have shown previously (13) that androgen-induced PSA promoter activity is inhibited in a dose-dependent manner by cotransfection with c-Jun expression plasmid. We hypothesized that c-Jun may potentially be involved in NSAID-mediated inhibition of AR. As shown in Fig. 2C, c-Jun protein was strongly induced by celecoxib and nimesulide at 24 h of treatment. Several studies (14–16), including ours, have shown that the transactivation functions of the AR as well as other steroid receptors can be affected by c-Jun. Therefore, these results strongly suggest that overexpressed c-Jun induced by celecoxib and nimesulide could interfere with AR-mediated up-regulation of PSA and hK2. Note that celecoxib at a relatively low concentration of 25 μM may not have an observable inhibitory effect on AR protein expression (Fig. 2A), but low concentrations of the NSAIDs could still increase c-Jun protein expression (Fig. 2C) and subsequently reduced the function of the AR, as evident in the transfections of Fig. 2B.

c-Jun is usually a short-lived protein, and it can be induced by many extracellular stimuli (17). In most cases, the induction is transient at early time of stimulation. However, the results in Fig. 2C show that c-Jun protein levels were elevated with 15 and 24 h of treatments, implying that the NSAIDs induced a prolonged overexpression of c-Jun.

Overexpression of c-Jun Inhibited the AR Promoter. To determine whether overexpression of c-Jun can affect the expression of the AR gene, we cotransfected a c-Jun expression construct with the two AR promoter reporter plasmids, AR promoter (−1380/+577)-pGL3 and AR promoter (−77/+84)-pGL3, respectively, in LNCaP cells. The result shown in Fig. 3B suggests that overexpression of c-Jun significantly inhibited the activity of both tested AR promoters.

Fig. 1. Effects of NSAIDs on the expression of PSA and hK2 proteins in prostate cancer cells ± Mib. LNCaP cells (A) and LAPC-4 cells (B) were treated with or without the indicated concentrations of celecoxib and nimesulide for 7 days. Spent media were collected and used for PSA (Hybritech) or hK2 (sandwich) immunoassay. PSA and hK2 values were normalized to growth response measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and expressed as a percentage of that of groups treated with Mib only. Error bars indicate the SE of four separate experiments.
Discussion

Recent studies (18, 19) have shown that AR expression appears in all stages of prostate cancer, regardless of the androgen-responsive status of the cancer. It has been shown that overexpression of many cofactors (e.g., caveolin-1, signal transducers and activators of transcription 3, and β-catenin) or coactivators (e.g., TIF2 and SRC-1) for the AR may enhance the function of the AR by low levels of androgens or by other non-androgen ligands (20–25). The presence of the AR and/or overexpression of cofactors or coactivators may provide a growth advantage for prostate cancer cells and represents one of the central targets for preventing/treating prostate cancer.

Many studies support a potential role of NSAIDs as chemopreventive agents (1–3). The molecular mechanism(s) by which NSAIDs exhibit anticancer activities is not completely understood. In the present study, we examined a panel of 11 commonly used NSAIDs, focusing on their effects on AR-mediated action in two androgen-responsive prostate cancer cell lines. We found that celecoxib and nimesulide exhibit much stronger activities than the other NSAIDs tested. The data strongly suggest that these two NSAIDs are potent inhibitors of AR function and that the induction of c-Jun expression/phosphorylation (Fig. 2C) plays an important role in mediating their effects. Clearly, our previous studies and those of others (13–16) demonstrated that c-Jun exhibits an inhibitory effect on AR protein activity. In addition, this study delineated that c-Jun can inhibit the transcription activities of the AR promoter. In the future, small interference RNA targeting c-Jun mRNA (26) or dominant negative c-Jun (27) might be used to further address the contribution of c-Jun to the function and expression of AR in prostate cancer cells.

c-Jun is a component of AP-1 protein complex and a member of the basic leucine zipper family of sequence-specific dimeric DNA-binding proteins (17). Previously, we and others have shown that stimulated overexpression of c-Jun protein can inhibit the function of the AR (13–16). Several studies have shown that the transactivation functions of the AR as well as other steroid receptors can be affected by c-Jun. The interaction...
between the AR and c-Jun is mediated through the DNA- and ligand-binding domains of AR and the leucine zipper region of c-Jun that may affect transactivation function of the AR (15). Other studies indicate that the transcriptional interference between AR and c-Jun is partly mediated through competition for DNA- and protein-protein interacting sites (28). Thus, the repression effect of c-Jun might be mediated through protein-protein interaction such that c-Jun does not need a direct contact with the AR promoter. Additional studies are required to resolve this issue.

It is worth mentioning that NSAIDs are a family of agents that inhibit the activity of COX-1 and COX-2, key enzymes converting arachidonic acid to PGs such as PGE₂ (29, 30). It has been suggested that the antineoplastic properties of NSAIDs may depend on their ability to inhibit PG synthesis. However, some reports indicate that this mechanism can only partially explain the antineoplastic properties of NSAIDs (31–33). Song et al. (32) showed that in COX-2 antisense prostate cancer cell clones, celecoxib still exhibits its antiproliferative activity independent of COX-2 expression. Our studies here have clearly shown that even at concentrations of approximately 25 μM, these two NSAIDs can significantly inhibit the function of AR. The low concentrations used (Table 1 and Figs. 1 and 2) are physiologically achievable for nimesulide or nearly achievable for celecoxib (32–34). However, note that these concentrations are far higher than that (at approximately 1 μM or lower levels) needed to inhibit COX-2 activity (33, 34). Therefore, this and our data seem to suggest that the AR-inhibiting effect mediated by these two NSAIDs is independent of their COX-2 inhibitory effects, although more direct evidence may be required to prove this point in the future study. Moreover, the effective concentrations of these two NSAIDs for inhibiting AR shown in the present study might be able to inhibit COX-1 activities; this is another issue that will need to be resolved in the future study.

In summary, our study shows strong evidence that celecoxib and nimesulide exhibit a novel property by which androgen action is blocked in prostate cancer cells. Traditionally, AR antagonists and the inhibitors of AR agonist synthesis are used to repress or reduce the function of the AR in hormone therapy for prostate cancer. However, both celecoxib and nimesulide can enhance the expression of c-Jun and further activate it by phosphorylation, through which the expression and function of the AR were inhibited. Our findings provide a strong rationale that celecoxib and nimesulide be potential agents for prostate cancer prevention and/or treatment.

References

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