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ORIGINAL ARTICLE

Use of Non-steroidal Anti-inflammatory Drugs for Chemoprevention of Inflammation-induced Prostate Cancer

İnflamasyonla Tetiklenen Prostat Kanserleşmesinin Önleyici Tedavisinde Non-streoidal Anti-inflamatuvar İlaç Kullanımı

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ABSTRACT

Objectives: Chronic inflammation has been known as one of the major causes of cancer progression and 25% of cancer cases initiate due to chronic inflammation according to epidemiologic data. It has been determined that chronic inflammation induces carcinogenesis through the abrogation of cell proliferation, apoptosis, and angiogenesis mechanisms. Therefore, it is believed that inhibition of inflammation-induced carcinogenic mechanisms is an efficient therapeutic strategy in drug development studies of cancer chemoprevention. It has also been observed that use of anti-inflammatory drugs reduces the incidence of cancer, and the risk of developing prostate cancer decreases 15-20% with regular use of aspirin and non-steroidal anti-inflammatory drugs (NSAID).

Materials and Methods: In this study, we investigated the effects of some clinically used NSAIDs on cellular mechanisms that play a role in inflammation-induced prostate carcinogenesis. Inhibition activities on the nuclear factor kappa-B signaling pathway, which activates tumorigenic mechanisms, as well as alterations on androgen receptor signaling, which regulates the proliferation of prostate cells, were investigated. In addition, protein kinase B (Akt) activation, which is stimulated a the inflammatory microenvironment, was examined.

Results: The results showed that anti-inflammatory agents alter the protein levels of androgen receptors as well as tumor suppressor NKX3.1, and might trigger an unexpected increase in $Akt^{(S473)}$ level, which induces tumorigenesis.

Conclusion: It is suggested that inflammatory pathways and prostate carcinogenesis-specific mechanisms should be taken into account for the use of anti-inflammatory drugs for chemoprevention of inflammation-induced prostate cancer.

Key words: NSAID, prostate cancer, inflammation, androgen receptor, NKX3.1

ÖZ

Amaç: Kronik enflamasyonun kanser gelişiminin önemli nedenlerinden biri olduğu ve epidemiyolojik verilere göre kanser olgularının %25'inin kronik inflamasyona bağlı olarak geliştiği bilinmektedir. Kronik inflamasyon, hücre proliferasyonu, apoptoz, anjiyogenez gibi mekanizmalarda bozukluklara yol açarak tümörleşmeyi tetiklemektedir. Bu nedenle kanseri önleyici ilaç geliştirme çalışmalarında, inflamasyon ile tetiklenen mekanizmaların inhibisyonununda etkin bir terapötik strateji olduğu düşünülmektedir. Klinik çalışmalarda da, birçok kanser tipinde anti-inflamatuvar ilaçların kanser insidansını azaltıcı etkisi belirlenmiş ve prostat kanserinde de düzenli aspirin veya non-streoidal anti-inflamatuvar ilaç (NSAID) kullananların prostat kanserine yakalanma riskinin yaklaşık %15-20 azaldığı saptanmıştır.

Gereç ve Yöntemler: Bu çalışma kapsamında, klinik kullanımdaki NSAID'lerden indometazin, sulindak, ibuprofen, naproksen, selekoksib ve nimesulidin inflamasyona bağlı prostat kanserleşmesinde rol oynayan moleküler mekanizmalardan bazıları üzerindeki etkileri araştırılmıştır. Tümörijenik mekanizmaları aktifleştiren nükleer faktör kappa B sinyal yolağı üzerindeki inhibisyon etkileri ve prostat hücrelerinin proliferasyonunu düzenleyen androjen reseptörü sinyal yolağı üzerindeki etkileri araştırılmıştır. Ayrıca inflamatuvar mikroçevrede etkinleşerek tümörleşmeye neden olan protein kinaz B (Akt) aktivitesindeki değişim de incelenmiştir.

Bulgular: Elde edilen sonuçlar, anti-inflamatuvar ajanların, androjen reseptörü ve tümör baskılayıcı NKX3.1'in protein seviyelerinde konsantrasyona bağlı bir değişime neden olduğu, ayrıca tümörleşmeyi tetikleyen $Akt^{(S473)}$ düzeyinde de beklenmedik bir aktivasyonu tetikleyebildiğini göstermiştir.

Sonuç: Anti-inflamatuvar ajanların, prostat inflamasyonu ile tetiklenen kanserleşme sürecini önleme amacıyla kullanımlarında yalnızca inflamatuvar yolakların değil prostat kanserleşmesine özgün mekanizmaların da dikkate alınması gereği ortaya konmuştur.

Anahtar kelimeler: NSAID, prostat kanseri, inflamasyon, androjen reseptörü, NKX3.1

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INTRODUCTION

Clinical studies have shown that use of anti-inflammatory drugs results in a decrease of the incidence of many types of cancer such as colon, prostate, and stomach cancer in particular.[1] Some anti-inflammatory drugs show antitumor activities through affecting pathways such as nuclear factor (NF) kappa-B (NF-κB), cyclooxygenase-2 (COX2), Wnt/B-catenin, Protein Kinase B (known as Akt or PkB), reactive oxygen and nitrogen species (RONS) production, and angiogenic vascular endothelial growth factor (VEGF), which function in cellular mechanisms such as proliferation, apoptosis, angiogenesis, and migration/invasion.[2-7] In addition, inflammation-related alterations of the androgen receptor (AR), NKX3.1, and Akt, which regulate cell proliferation in co-operation, have a significant role in prostate tumorigenesis.[8-10]

The negative correlation between the use of anti-inflammatory drugs and cancer incidence proves that active inflammation supports carcinogenesis, and anti-inflammatory drugs can prevent inflammation-related tumorigenesis.[4,5] Inflammation leads to neoplastic transformation by altering gene expression levels of oncogenes and tumor suppressors. In addition, these genetic changes influence the expression patterns of many inflammation-related genes and result in the recruitment of inflammatory cells in the tumor microenvironment. These alterations cause tumor cells to produce free radicals, and inflammatory cytokines create a feedback loop that supports carcinogenesis.[11-13]

Androgen receptors have a critical role in the survival of prostate cells. However, AR signaling is also abrogated during inflammation. Activation of AR signaling supports the development of prostate adenocarcinomas because prostate cells depend on AR for proliferation. On the other hand, androgen ablation therapy leads to a tumor reduction at the beginning; however, paradoxically, it induces and accelerates the transition to castration-resistant-stage prostate cancer. In this stage, prostate cells do not depend on AR presence to survive and proliferate.[14,15]

NKX3.1 is a tumor suppressor protein whose expression is lost in most primary prostate cancers.[16,17] Loss of NKX3.1 function is observed through the related chromosome deletion and enhanced proteasome degradation induced by pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α.[18] Further, it has been shown that NKX3.1 expression was alleviated in proliferative inflammatory atrophy and prostatic intraepithelial neoplasia.[10,19]

In the present study, we aimed to investigate the potential chemo-preventative effects of commonly used NSAIDs on NF-κB signaling and inflammation-induced degradations of AR and NKX3.1, which have a significant role on inflammation-related prostate tumorigenesis.

EXPERIMENTALS

Cell culture and treatments

LNCaP cells were obtained from American Type Culture Collection (ATCC Manassas, VA) and propagated using RPMI 1640 supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL). Synthetic androgen R1881 was used at 10 nm concentration. A specific NF-κB inhibitor (BAY11-7082) and celecoxib were purchased from Biovision. Indomethacin, sulindac, ibuprofen, naproxen, and nimesulide were purchased from Sigma and used at the indicated concentrations determined according to the assay approach.

Conditioned media (CM) collection and measurement of cytokines in CM

The U937 monocyte cell line was cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. To achieve macrophage differentiation and cytokine production, cells ($8x10^6$) were grown in 75 cm^2 culture flasks for 2 h prior to treatment. Next, phorbol 12-myristate 13-acetate was added at a final concentration of 16 nM for 16 h, and adherent clusters were followed. Cells were washed twice, and 20 mL of fresh medium was then added. After allowing the cells to rest for 2-3 h, lipopolysaccharide was added (10 ng/mL), and the cells were incubated for 24 h. Finally, the supernatant [conditioned medium (CM)] was collected and filtered (0.2 µm) for further use.

Before feeding the LNCaP cells with CM, TNF-α (Invitrogen), IL-6, and IL-1β (Boster Biological Technology Co., US) levels were assessed using an enzyme-linked immunosorbent assay in accordance with the manufacturer's recommendations. Finally, CM with known concentrations was used to induce an inflammatory microenvironment in cell culture.

Antibodies

The antibodies were purchased from the manufacturers as follows: p-Akt$^{(S473)}$ from SCBT; AR antibody from Millipore; B-actin antibody from Sigma-Aldrich; HRP-anti-mouse and HRP-anti-rabbit secondary antibodies were purchased from Amersham BioSciences UK, and used as recommended. NXK3.1 antibody was kindly supplied by Prof. Saatcioglu from the Oslo Biotechnology Center, Norway.

NF-κB luciferase reporter assay

LNCaP cells (10^4) were seeded in 96-well culture plates and incubated for 48 h. Cell culture media was replaced with serum/antibiotic-free culture medium 2 h before the transfection. Negative and positive control vectors (100 ng/mL), and NF-κB (100 ng/mL) reporter vector (SA biosciences Cignal NF-κB reporter luc kit-CCS-013L) were transfected using fugene HD transfection reagent (Roche). Normal culture medium was added onto cells 5 h after transfection and incubated for 24 h. Treatments were performed for 4 h and cells were collected with passive lysis buffer according to the recommendations of dual-luciferase reporter assay kit (Promega, UK). Renilla/firefly luciferase activity measurements were obtained using a luminometer (Thermo) according to the manufacturer's protocol.

Cell lysis, protein extraction and blotting

For protein extraction, cells were grown in 6 cm plates (Sarstedt, Germany) and washed once with PBS prior to cell lysis. Next,

250 µL ice-cold modified RIPA buffer [10 mM Tris Cl (pH 8.0), 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate, 1 mM EDTA, 1 mM EGTA and 140 mM NaCl] containing both protease and phosphatase inhibitors was added to the plates, and the cells were then collected into Eppendorf tubes using a cell scraper. The lysates were sonicated for 20 seconds (25% power, 0.5 cycles), centrifuged at 12.000 g for 10 min at 4˚C, and the cleared supernatants were transferred into new tubes. The protein concentration was determined using a BCA assay (Sigma, UK). Western blots were performed under standard conditions using 50 µg of protein lysate per lane. First, the proteins were separated on a 10-12% SDS-PAGE gel and transferred to a PVDF membrane (Amersham BioSciences, UK) using a wet transfer blotter. The PVDF membrane was blocked with 5% dry milk in TBS-T (Tris-Borate-Saline solution containing 0.1% Tween 20), and then primary and secondary antibody incubations were performed using TBS-T containing 0.5% dry milk or 5% BSA at RT for 1 h or at 4˚C overnight. The membranes were developed using ECL plus reagent (Amersham BioSciences, UK) for 5 min and photographed using Kodak X-ray films in a dark room.

Statistical analysis

Student's t test was applied to assess the statistical significance between pairs when necessary using Microsoft Excel program.

RESULTS

Relative NF-κB inhibition activities of the selected NSAIDs

As inhibition of the NF-κB pathway is a key strategy to protect cells against inflammation-induced tumorigenic alterations, commonly used NSAIDs were investigated for their inhibition activity of the NF-κB signaling pathway using the luciferase reporter method. LNCaP cells were seeded 10^4/well on 96-well plates and incubated for 48 h. The cells were then transfected with NF-κB luciferase reporter and control vectors for 24 h and treatment were performed as 25 ng/mL TNF-α and indicated concentrations of anti-inflammatory drugs (sulindac 5 µM, naproxen 12.5 µM, ibuprofen 50 µM, nimesulide 50 µM, indomethacin 100 µM, celecoxib 50 µM) for 4 h. NF-κB activity was measured for each well and relative luciferase activity was determined as fold change of TNF-α induced cells to non-induced cells after firefly/renilla normalization. NF-κB activity was induced to 179-fold using TNF-α treatment and suppressed to 63 (sulindac), 76 (naproxen), 128 (ibuprofen), 77 (nimesulide), 71 (indomethacin), and 41 fold (celecoxib) at indicated concentrations. An NF-κB specific inhibitor (BAY11-7082) (5 µM) was used as a positive control, which led to a 98-fold decrease in NF-κB signaling activity (Figure 1).

Chemo-preventative activities of NSAIDs on inflammation-related prostate tumorigenesis

Loss of NKX3.1 in proliferative inflammatory atrophy regions was shown in previous studies. Preventing the loss of NKX3.1 in inflammatory conditions could be a useful strategy for the inhibition of tumorigenic alterations in prostate cells because it is a tumor suppressor that functions in response to DNA damage and oxidative stress regulation. Therefore, in order to understand whether NSAID treatments could prevent inflammation-induced NKX3.1 proteasomal degradation, the effects of NSAID treatments on NKX3.1 expression were investigated in LNCaPs. Cells were treated with CM (125 pg/mL TNF-α including conditioned media) for 24 h w/wo NSAIDs, which were added 2 h before CM. A decrease in NKX3.1 protein level due to cytokine-induced degradation was observed after CM as detected in our previous studies.[9,10] It was also determined that indomethacin, celecoxib, and nimesulide at their NF-κB inhibitory concentrations enhanced this degradation. NKX3.1 levels remained the same with sulindac, ibuprofen, and naproxen. None of the agents led NKX3.1 to be protected from degradation in inflammatory conditions, although NF-κB related pro-inflammatory activation was suppressed as shown by relative luciferase activity. In addition, activation of PI3K/Akt signaling, which induces proliferation, was also checked because NSAIDs could affect the activation of Akt phosphorylation. It has been determined that CM treatment increased p-Akt$^{(S473)}$ levels, consistent with our previous studies.[8] Sulindac and naproxen were found to suppress phosphorylation to its basal levels in inflammatory conditions (Figure 2).

AR signaling has a regulatory role for prostate cell proliferation. Moreover, AR signaling and tumor suppressor AR and NKX3.1 are key factors of inflammation-related prostate tumorigenesis. Therefore, in order to enlighten the dose-dependent effects of the gents on their expressions, LNCaPs were treated for 24 h. Indomethacin led to a concentration-dependent decrease on AR and NKX3.1 levels. Sulindac and ibuprofen treatments resulted in an unchanged expression profile of NKX3.1. Naproxen treatment enhanced NKX3.1 protein levels up to 3 and 6 µM concentrations, and did not change AR levels. On the other hand, celecoxib and nimesulide alleviated NKX3.1 expression in

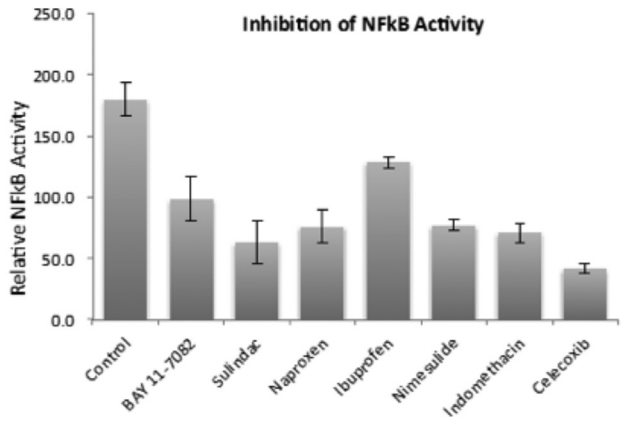


Figure 1. Relative NF-κB inhibition activities of NSAIDs. 10^4 LNCaP cells were transfected with NF-κB lusiferase reporter vector and incubated for 48 h. Cells were treated with 40 ng/mL TNF-α and anti-inflammatory agents for 4 h with the concentrations that Sulindac 5 µM, Naproxen 12.5 µM, Ibuprofen 50 µM, Nimesulide 50 µM, Indomethacin 100 µM, Celecoxib 50 µM. Specific NF-κB inhibitor BAY11-7082 was used as a positive control with a concentration of 5 µM. Dual luciferase activity was measured and relative luciferase activity was calculated as fold change of luciferase activity of TNF-α treated cells versus untreated cells after firefly/renilla normalization. Error bars represent the standard deviation of 3 replicates, $p<0.001$

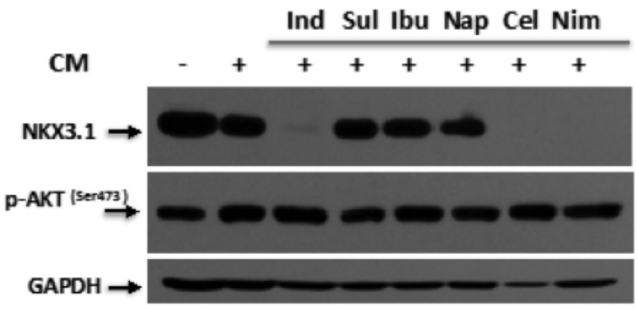


Figure 2. Change of tumor suppressor NKX3.1 expression due to NSAIDs in inflammatory microenvironment. $6x10^5$ LNCaP cells were seeded and incubated for 48 h. NSAIDs were applied onto cells 2 h before CM treatment and cell were incubated further 24 h after CM. NKX3.1 and p-Akt$^{(S473)}$ expressions were detected by immunoblotting. GAPDH was used as a loading control. CM: 125 pg/mL TNF-α including conditioned media, Indomethacin: 100 μM, Sulindac: 10 μM, Ibuprofen: 50 μM, Naproxen: 12.5 μM, Celecoxib: 50 μM, Nimesulide: 50 μM.

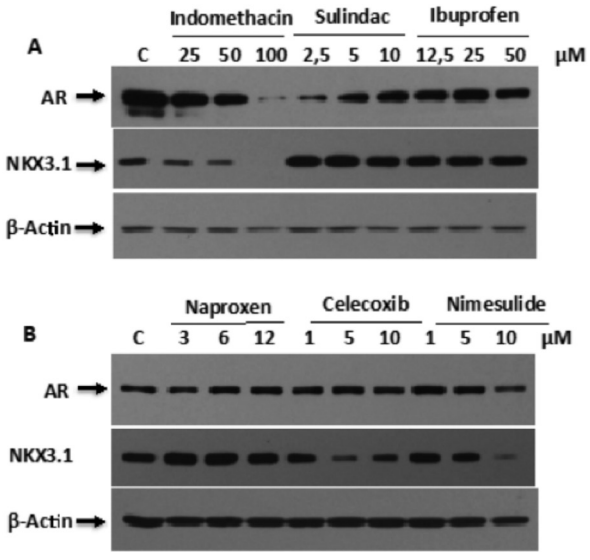


Figure 3. Alterations on AR and NKX3.1 expressions after NSAID treatments. $6x10^5$ LNCaP cells were seeded and incubated for 48 h. NSAIDs at indicated concentrations were applied onto cells for 24 h and expressions of AR and NKX3.1 were detected by immunoblotting. B-actin was used as a loading control

a concentration-dependent manner. AR protein levels remained largely stable with these agents (Figure 3a, b).

Finally, activities of anti-inflammatory drugs on the recovery of AR and NKX3.1 in the inflammatory microenvironment were investigated. LNCaP cells were treated with indicated concentrations of agents (26 h) in the absence and presence of CM (24 h) and synthetic androgen R1881 (26 h). Indomethacin severely enhanced NKX3.1 degradation during inflammation; however, stimulation of AR signaling enhanced the expressions of both AR and NKX3.1 in the presence of indomethacin. Sulindac, ibuprofen, and naproxen treatments were determined to partially protect NKX3.1 from degradation when compared with other agents. Further, degradation of NKX3.1 was not prevented by celecoxib and nimesulide in inflammatory conditions. In

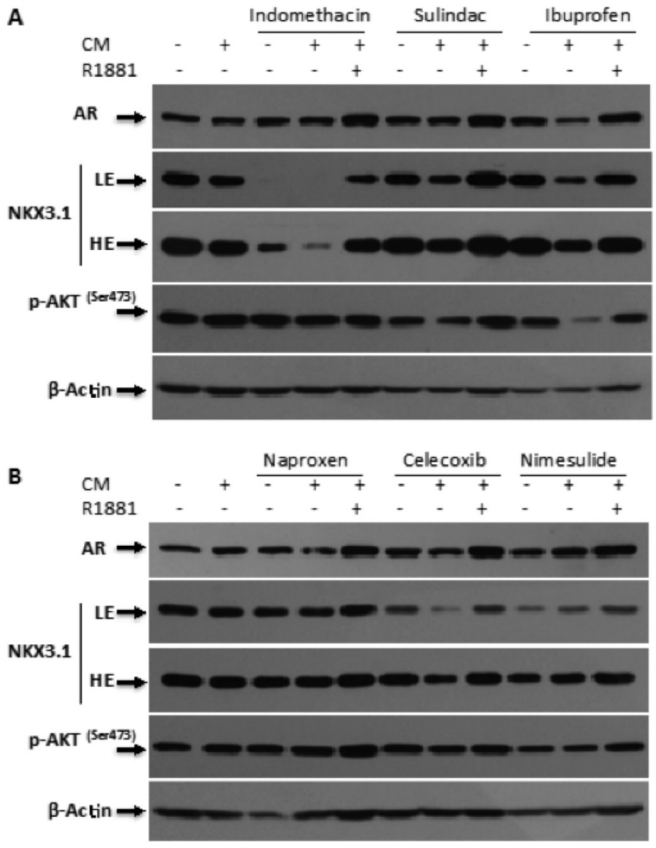


Figure 4. Effects of NSAIDs on AR signaling in inflammatory microenvironment. $6x10^5$ LNCaP cells were seeded and incubated for 48 h. NSAIDs and R1881 (10 nM) were applied onto cells 2 h before CM treatment and cell were incubated further 24 h after CM. NKX3.1 expression was detected by immunoblotting. B-actin was used as a loading control. CM: 125 pg/mL including conditioned media, Indomethacin: 50 μM, Sulindac: 5 μM, Ibuprofen: 12.5 μM, Naproxen: 6 μM, Celecoxib: 1 μM, Nimesulide: 1 μM

the presence of anti-inflammatory drugs, activation of AR signaling by R1881 treatment led to increased AR and NKX3.1 protein levels, even in the inflammatory microenvironment, as expected. Sulindac, ibuprofen, and nimesulide were detected to inhibit p-Akt$^{(S473)}$ phosphorylation even after CM treatment (Figure 4a, b).

DISCUSSION

Inflammation and the subsequent unbalanced anti-inflammatory response is known to activate tumorigenic mechanisms.[20-22] Previous studies showed that use of anti-inflammatory drugs reduced the incidence of many types of cancer such as prostate and colon cancer[23-25], as such, the development of therapeutic strategies for the inhibition inflammation-related tumorigenic mechanisms is a useful approach in the prevention of inflammatory diseases and cancer chemoprevention.[26-28] In this study, activities of NSAIDs on NF-κB inhibition and the AR signaling pathway were investigated in order to establish the protective concentrations of anti-inflammatory drugs that inhibit tumorigenic alterations in prostate cells.

Inhibition of AR and NKX3.1 degradation during inflammation has been suggested as a key strategy to protect prostate cells

from deregulated oxidative stress and enhanced proliferation. Although inhibition of AR signaling is the main strategy of primary prostate tumors through chemical castration with anti-androgens, it has been also reported that loss of AR signaling results in insufficient oxidative stress regulation with subsequent oxidative DNA damage and genomic heterogeneity, leading to castration-resistant prostate cancer.[29,30] Therefore, protective concentrations of NSAIDs on AR and NKX3.1 protein stabilities were investigated and sulindac, ibuprofen, and naproxen were found as the most efficient molecules for maintaining regulatory AR signaling and NKX3.1 function. However, it was also determined that increasing concentrations of the agents were not always correlated to AR and NKX3.1 recovery, possibly because of the cytotoxic effects of relatively higher concentrations. Therefore, it was suggested that as AR and NKX3.1 were survival factors for prostate cells that degrade under cytotoxic cellular conditions; concentrations that suppress cell proliferation via NF-κB pathway and effect cellular viability were not optimal to protect cells from AR and NKX3.1 degradation. In addition, enhanced Akt phosphorylation due to inflammation was mostly suppressed by sulindac (5 μM), ibuprofen (12.5 μM), and nimesulide (1 μM). Because sulindac, ibuprofen, and naproxen showed consistent protection for AR and NKX3.1 protein levels at their anti-inflammatory concentrations, it is concluded that use of this agents for the cure of prostatitis favors protecting cells from loss of AR signaling induced by inflammatory microenvironment.

Surprisingly, it is known that NSAIDs such as indomethacin[31] can activate Akt signaling, which enhances S473 phosphorylation, consistent with our results. Increased p-Akt$^{(S473)}$ levels result in NKX3.1 suppression[9]; the negative correlation between NKX3.1 recovery and Akt phosphorylation in our results suggests that NSAIDs that lead to unexpected activation of Akt signaling such as indomethacin should be taken into account for their effects on tumor suppressor NKX3.1. On the other hand, sulindac was shown to suppress this activation in our results, as well as in a previous study[32], which supports its inhibition potential on inflammation-induced tumorigenic events. Celecoxib was also reported to induce apoptosis via inhibition of Akt activation.[33] However, because celecoxib showed a severe suppression on NKX3.1 protein levels at its NF-κB inhibitory concentration, and also mild protection on AR and NKX3.1 without suppression of Akt phosphorylation at its lower concentrations in our results, we concluded that celecoxib was not an effective candidate among the commonly used selective COX2 inhibitors for cancer chemoprevention. Ibuprofen also has a suppression activity on the proliferation of prostate cancer cells[34] according to the literature. It is suggested that significant suppression of p-Akt$^{(S473)}$ levels could account for this effect on prostate cells. In addition, it is suggested that this effect results from the stabilization of AR and NKX3.1 by androgens in the presence of NSAIDs because it has been also reported that the presence of dihidrotestosterone enhances the apoptotic effects of anti-inflammatory agents.[35] However, the concentration of the agents was observed to be critical in balancing anti-inflammatory activity and further carcinogenic alterations such as protein stability of AR, as well as NKX3.1 and Akt activation, as found in our study.[36]

We suggest that these results are significant for the selection of the best therapy among many anti-inflammatory therapy alternatives for prostatic inflammation because our findings show the secondary effects of commonly used NSAIDs on carcinogenesis-related AR signaling abrogation and Akt activation in prostate cells.

CONCLUSION

Inflammation-related changes in cellular mechanisms could lead to carcinogenesis. Therefore, these alterations should be reversed as a strategy for the chemoprevention of carcinogenesis. In the case of inflammation-induced prostate carcinogenesis, functional protection of AR signaling and tumor suppressor NKX3.1 is a valuable strategy that should be taken into consideration to achieve the right therapy combination for prostatic inflammation.

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