Prostaglandin receptor EP3 mediates growth inhibitory effect of aspirin through androgen receptor and contributes to castration resistance in prostate cancer cells

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Abstract
Although numerous epidemiological studies show aspirin to reduce risk of prostate cancer, the mechanism of this effect is unclear. Here, we first confirmed that aspirin downregulated androgen receptor (AR) and prostate-specific antigen in prostate cancer cells. We also found that aspirin upregulated prostaglandin receptor subtype EP3 but not EP2 or EP4. The EP3 antagonist L798106 and EP3 knockdown increased AR expression and cell proliferation, whereas the EP3 agonist sulprostone decreased them, indicating that EP3 affects AR expression. Additionally, EP3 (PTGER3) transcript levels were significantly decreased in human prostate cancer tissues compared with those in normal human prostate tissues, suggesting that EP3 is important to prostate carcinogenesis. Decreased EP3 expression was also seen in castration-resistant subtype CxR cells compared with parental LNCaP cells. Finally, we found that aspirin and EP3 modulators affected prostate cancer cell growth. Taken together, aspirin suppressed LNCaP cell proliferation via EP3 signaling activation; EP3 downregulation contributed to prostate carcinogenesis and to progression from androgen-dependent prostate cancer to castration-resistant prostate cancer by regulating AR expression. In conclusion, cyclooxygenases and EP3 may represent attractive therapeutic molecular targets in androgen-dependent prostate cancer.

Key Words
- androgen receptor
- aspirin
- castration-resistant prostate cancer
- cyclooxygenase
- EP3
- prostate cancer

Introduction
Prostate cancer is the most commonly diagnosed non-skin cancer among men in developed countries (Jemal et al. 2008). In its early stages, prostate cancer cell growth is androgen dependent, and androgen deprivation therapies cause prostate tumor regression. Unfortunately, the majority of prostate cancers eventually transit to castration-resistant prostate cancer (CRPC). Androgen receptor (AR) plays a vital role in cell growth and survival of both androgen-dependent prostate cancer and CRPC. Especially, in CRPC, AR is thought to be inappropriately activated under the condition of castration levels of androgens (Kung & Evans 2009); AR inhibition represses tumor growth in both androgen-dependent prostate cancer and CRPC (Chen et al. 2003, Scher & Sawyers 2005).
Inflammation by various insults is thought to be a major cause and promoter of various cancers including prostate cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenases (COXs) and suppress prostaglandin (PG) synthesis, thus fighting inflammation (Majima et al. 2003, Wang & DuBois 2010). NSAIDs have been reported to reduce risk of developing some solid tumors, including breast, colon, lung, and prostate cancer (Harris 2009), as well as exert antitumor effects. The COX2 inhibitor celecoxib decreased patients’ rising prostate-specific antigen (PSA) rates after radical therapies (Pruthi et al. 2004, Smith et al. 2006). NSAIDs may suppress prostate cancer incidence and tumor development by suppressing AR transcription and promoting apoptosis in prostate cancer cells (Lim et al. 1999, 2003, Pan et al. 2003). However, the precise mechanism of NSAIDs’ preventative and therapeutic effects is not well understood.

COXs have two distinct isoforms: COX1 and COX2. COX1 is expressed constitutively in several tissues, whereas COX2 is induced by cytokines, mitogens, and tumor promoters (Katori & Majima 2000, Gupta & DuBois 2001, Subbaramaiah & Dannenberg 2003), resulting in enhanced synthesis of PGs in inflamed and neoplastic tissues (Sharon et al. 1978, Bennett 1986, Rigas et al. 1993). The expression of COX2 is higher in prostate cancer tissues than in benign prostate tissues (Gupta et al. 2000, Yoshimura et al. 2000) and increases as differentiation attenuates (Shappell et al. 2001). PGE2 produced by COXs is reportedly crucial to angiogenesis and oncogenesis in prostate cancer (Jain et al. 2008). Consistent with the finding of COX2 overexpression in prostate cancer, PGE2 content is also almost tenfold higher in malignant prostatic tissues than in benign prostatic tissues (Chaudry et al. 1994). PGE2 acts through four G-protein-coupled receptors: EP1, EP2, EP3, and EP4. EP2 and EP4 bind to stimulative G proteins and increase intracellular cAMP whereas EP3 couples to an inhibitory G protein and decreases cAMP. EP1 increases intracellular calcium (Cha & DuBois 2007). However, EP1 was not detectable in human prostate cancer cell lines (Wang & Klein 2005). On the other hand, EP3 expression was decreased compared with normal mucosa in colon cancer of mice, rats, and humans (Shoji et al. 2004). Furthermore, Macias-Perez et al. revealed that mouse EP3 reduced tumor cell proliferation and tumorigenesis in vivo (Macias-Perez et al. 2008) and contributed to growth inhibition or cellular senescence (Fulton et al. 2006). Taken together, these results suggest that EP3 signaling could be the basis of a novel anticancer therapy. However, the contribution of EP3 in prostate cancer is not well understood.

To establish the rationale of aspirin use in chemoprevention of prostate cancer, we investigated the mechanism of aspirin’s effect on AR expression in prostate cancer and in preventing prostate transformation, using human prostate cancer cells and tissues. Furthermore, we tried to show the therapeutic effect of aspirin, as well as its downstream target on prostate cancer, including CRPC, for which effective therapeutics are limited.

Materials and methods

Cell culture

Human prostate cancer DU145 (MEM), LNCaP (RPMI-1640), and 22Rv1 (RPMI-1640) cells were purchased from the American Type Cell Collection (Manassas, VA, USA) and were cultured under media in parenthesis purchased from Invitrogen supplemented with 10% fetal bovine serum. LNCaP cells that propagated between 10 and 40 times were used. Castration-resistant derivatives of LNCaP cells (LNCaP-CxR cells, referred to as CxR cells) were established and maintained as described previously (Shiota et al. 2009b). All cell lines were maintained in a 5% CO2 atmosphere at 37°C.

Antibodies and drugs

Antibody against AR (sc-815) was purchased from Santa Cruz Biotechnology. Anti-EP2, anti-EP3, and anti-EP4 antibodies, sulprostone and PGE2, were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Anti β-actin and anti-PSA antibodies were purchased from Sigma and Epitomics, Inc. (Burlingame, CA, USA) respectively; L798106 was purchased from Sigma.

RNA isolation and RT

Total RNA was prepared from cultured cells using an RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 1.0 μg total RNA using a Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer’s protocol.

Quantitative real-time PCR

Synthesized cDNA was diluted to 1:2 ratio; 2.0 μl of the diluted sample was used. TissueScan Prostate Cancer Tissue qPCR array III (HPRT303) was purchased from OriGene (Rockville, MD, USA). This product contains first-strand cDNAs prepared from 48 human prostate tissues,
including both malignant and healthy controls. These 48 cDNAs had been normalized against β-actin by RT-PCR and arrayed onto PCR plates. Quantitative real-time PCR with TaqMan Gene Expression Assay (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems) was performed using an ABI 7900HT System; GAPDH values were used for normalization. Results are representative of at least three independent experiments.

**Western blot analysis**

Western blot analysis was performed as described previously (Kashiwagi et al. 2010). To prepare whole cell lysates, cells were sonicated for 20 s with buffer-Y. Whole cell lysates (30 μg) were separated by SDS–PAGE and transferred onto PVDF membranes. Western blot analysis was performed using appropriately diluted antibodies. The membrane was developed using a chemiluminescence protocol (GE Healthcare, Waukesha, WI, USA). Images were obtained using an image analyzer (LAS-3000 Mini; Fujifilm, Tokyo, Japan).

**Knockdown analysis using siRNAs**

Knockdown analysis using siRNAs was performed as described previously (Kashiwagi et al. 2010). The following double-stranded RNA 25 bp oligonucleotides were commercially generated (Invitrogen): EP3 (PTGER3) siRNA: siEP3 #2, 5′-CGAACAGCUAUAAAGAAGAAGUUGC-3′ (sense) and 5′-GCAACUUCUUCAUAUGCGUGUCG-3′ (antisense); siEP3 #3, 5′-UUACACGAGGUAACCCAGGAUC-3′ (sense) and 5′-GAUCCUUGGGUUAACCUCGCU-GUUAA-3′ (antisense). LNCaP cells were transfected with various amounts of the siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

**Cytotoxicity analysis**

Cytotoxicity analysis was performed as described previously (Kashiwagi et al. 2010). LNCaP and CxR cells...
(2×10^4) were seeded into 96-well plates. The following day, various concentrations of aspirin were applied in medium. After 48 h, the surviving cells were stained with the Alamar Blue assay (TREK Diagnostic Systems, Cleveland, OH, USA) for 180 min at 37 °C. Absorbance of the wells was measured using a plate reader (ARVO MX; Perkin Elmer, Inc., Waltham, MA, USA).

Cell proliferation assay

Cell proliferation assay was performed as described previously (Shiota et al. 2008, 2009a). Briefly, LNCaP cells (2.5×10^5) were seeded into 12-well plates and transfected with the indicated siRNA or added indicated drugs under androgen-deprived conditions. After 96 h, the cells were harvested with trypsin and counted using a cell counter (Beckman Coulter, Fullerton, CA, USA). The results were normalized by cells transfected with control siRNA or treated with vehicle at 96 h and are representative of at least three independent experiments.

Statistical analysis

The Mann–Whitney U test was used for statistical analysis. Significance was set at P ≤0.05.

Results

Aspirin reduces AR and PSA expression in prostate cancer cells

To confirm aspirin’s effects on AR expression, we examined mRNA and protein levels of AR in LNCaP cells after aspirin treatment. Both AR and PSA mRNA, and both AR and PSA protein levels, were decreased by aspirin treatment in a dose-dependent manner (Fig. 1A and B respectively). Aspirin (1 mM) also decreased AR and PSA protein in a time-dependent manner (Fig. 1C).

PGE2 does not induce AR expression in prostate cancer cells

As aspirin – a COX inhibitor – decreases PGE2 levels, we supposed that aspirin might regulate AR expression through PGE2. However, when we examined AR expression after exposure to PGE2, we found that PGE2 did not affect AR mRNA or protein levels in LNCaP cells (Fig. 2A and B).

Aspirin induces EP3 expression in prostate cancer cells

Based on the results mentioned earlier, we hypothesized that COX-independent pathway regulates AR expression in prostate cancer cells and speculated that aspirin may affect expression of PG receptors. We then examined effects of aspirin on PG receptor subtypes, EP2, EP3, and EP4. Surprisingly, aspirin reduced EP2 expression and induced EP3 expression but not EP4 in dose- and time-dependent manners in LNCaP cells (Fig. 3A and B). We also examined aspirin’s effect on expressions of AR and PG receptors using another androgen-sensitive prostate cancer cell line 22Rv1 cells. Expectedly, aspirin induced EP3 expression concurrently with AR suppression, similar to LNCaP cells; however, EP2 expression was only marginally reduced by aspirin treatment in 22Rv1 cells (Fig. 3C). Consistently, AR transactivity represented by PSA transcription in 22Rv1 cells was also decreased by aspirin treatment in a dose-dependent manner (data not shown).
Furthermore, EP3 expression was similarly induced also in AR-null DU145 cells. Expression of PG receptors in LNCaP cells was not influenced by exposure to PGE2 (Fig. 3D), indicating that PGE2 did not affect PG receptor expression as well as AR did. These findings indicate that aspirin affects AR expression through PG receptor expression, but not through PGE2. Because aspirin treatment commonly increased EP3 expression in two prostate cancer cell lines, LNCaP and 22Rv1, we thereafter focused on EP3 as a regulator of AR expression, but not EP2 and EP4.

**EP3 regulates AR expression in prostate cancer cells**

We initially investigated the effects of EP3 on AR expression using pharmacological manipulation. The EP3 agonist sulprostone suppressed AR mRNA and AR and PSA protein expressions in LNCaP cells (Fig. 4A and B). Inversely, the EP3 antagonist L798106 increased AR mRNA (Fig. 4C) and AR and PSA protein expressions (Fig. 4D). We then employed a knockdown method using EP3-specific siRNAs, which successfully suppressed EP3 expression and upregulated AR expression (Fig. 4E). Thus, it was confirmed that EP3 negatively regulates AR expression, using both pharmacological and knockdown methods.

**EP3 expression decreases in clinical samples of prostate cancer and in castration-resistant cells**

Because EP3 expression was negatively correlated with AR expression, which is known to promote prostate cancer development and progression, we hypothesized that EP3 is decreased in prostate cancer samples. Therefore, we examined EP3 expression in normal prostate tissues and prostate cancer tissues using a prostate cancer tissue array containing cDNAs derived from nine normal prostate tissues and 39 prostate cancer tissues and real-time quantitative PCR. EP3 expression was remarkably downregulated in prostate cancer compared with normal prostate tissues (Fig. 5A), suggesting that EP3 downregulation may contribute to cancerous changes in prostate tissues.

Next, because AR expression is closely implicated in prostate cancer progression to CRPC, we examined

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**Figure 3**

Aspirin induces EP3 expression in LNCaP and 22Rv1 cells. (A) LNCaP cells were cultured with indicated concentrations of aspirin for 48 h; cell lysates (30 µg) were analyzed for EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control. (B) LNCaP cells were cultured with 1 mM aspirin for indicated times; cell lysates (30 µg) were analyzed for EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control. (C) 22Rv1 cells were cultured with indicated concentrations of aspirin for 48 h; cell lysates (30 µg) were analyzed for AR, EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control. (D) LNCaP cells were cultured with indicated concentration of PGE2 in serum-free medium for 48 h; cell lysates (30 µg) were analyzed for EP2, EP3, and EP4 using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control.
expressions of PG receptors in CPRC using castration-resistant LNCaP derivative CxR cells. Next, because AR expression is closely implicated in prostate cancer progression to CRPC (Shiota 2011), we examined expressions of PG receptors in CPRC using castration-resistant LNCaP derivative CxR cells (Shiota 2009b). EP3 expression was downregulated concurrently with upregulation of AR in CxR cells compared with LNCaP cells (Fig. 5B); however, EP2 and EP4 expressions were upregulated, suggesting that EP3 downregulation is also involved in prostate cancer progression to CRPC. Accordingly, neither EP3 agonist sulprostone nor EP3 antagonist L798106 affected AR expression at both mRNA and protein levels (Fig. 5C and D), indicating an abolished EP3 signaling in CRPC.

Figure 4
The EP3 agonist sulprostone decreases AR expression and the EP3 antagonist L798106 increases AR expression in LNCaP cells. (A) LNCaP cells were cultured with indicated concentrations of sulprostone for 24 h. The mRNA level of AR was analyzed using quantitative real-time PCR. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ± S.D. *P < 0.05 (compared to untreated LNCaP cells). (B) LNCaP cells were cultured with indicated concentrations of L798106 for 48 h; cell lysates (30 µg) were analyzed for AR and PSA using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control. (C) LNCaP cells were transfected with 40 nM of control siRNA, EP3 siRNA #2, or EP3 siRNA #3. At 72 h after transfection, whole cell extracts (30 µg) were analyzed for EP3 and AR using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control.
EP3 signaling modulates cell growth of prostate cancer

Based on the result mentioned earlier, we hypothesized that proliferation of prostate cancer under castration conditions is affected by EP3 signaling. We therefore examined LNCaP cell proliferation under castration conditions after modulating EP3 signaling by pharmacological and knockdown methods. Surprisingly, the EP3 agonist sulprostone suppressed LNCaP cell growth under an androgen deprivation medium in a dose-dependent manner (Fig. 6A), while the EP3 antagonist L798106 increased (Fig. 6B). In addition, EP3 knockdown accelerated LNCaP cell growth in an androgen deprivation medium after 96 h (Fig. 6C). Thus, these data indicate that EP3 signaling is a critical regulator of prostate cancer growth under androgen-deprived conditions.

Figure 5
EP3 expression in prostate cancer samples and CxR cells. (A) Expression of EP3 mRNA in normal prostate and prostate cancer was determined. The log2 of normalized (to β-actin) expression values relative to the mean expression of the normal is presented in normal prostate vs prostate cancer. The line in the middle represents the median; bars, ± s.d. *P < 0.05 (compared to normal prostate). (B) Whole cell extracts (30 μg) from LNCaP and CxR cells were analyzed for AR, EP2, EP3, and EP4 using SDS–PAGE and western blot analysis with specific antibodies. The anti-β-actin antibody was used as a loading control. (C) CxR cells were cultured with indicated concentrations of sulprostone for 24 h. The mRNA level of AR was analyzed using quantitative real-time PCR. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. Cell lysates (30 μg) were analyzed for AR using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control. (D) CxR cells were cultured with indicated concentrations of L798106 for 24 h. The mRNA level of AR was analyzed using quantitative real-time PCR. All values represent means of at least three independent experiments. Boxes, mean; bars, ±s.d. Cell lysates (30 μg) were analyzed for AR using SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control.
Next, we examined aspirin’s effect on the viability of prostate cancer cells. Expectedly, aspirin treatment suppressed LNCaP cell viability. However, surprisingly, aspirin had less effect on the viability of CxR cells (Fig. 6D). This discrepancy between LNCaP and CxR cells may result from downregulated EP3 expression in CxR cells (Fig. 5B), suggesting that aspirin does not exert therapeutic effects after progression to CRPC.

Discussion

NSAIDs are thought to exert anticancer activities through both COX-dependent and -independent pathways. COX2 has been shown to affect cancer development by promoting cell division, inhibiting apoptosis, stimulating neoangiogenesis, and altering cell adhesion (Grösch et al. 2006). Inversely, inhibition of COX2 activity is supposed to block these activities and exert tumor-preventative effects. Thus, through COX-dependent pathway, NSAIDs can show anticancer effects. Conversely, a selective COX2 inhibitor, celecoxib, was shown to target COX-independent pathway such as Ca^{2+} ATPase, protein-dependent kinase 1, and cyclin-dependent kinases, resulting in inhibitions of antiapoptosis cell cycle progression, angiogenesis, and metastasis (Hwang et al. 2002, Grösch et al. 2006). Furthermore, NSAIDs inhibited cell growth of COX2-null colorectal cancer (Chan et al. 1998), indicating a COX2-independent anticancer effect of NSAIDs. Similarly, in prostate cancer cells, Patel et al. (2005) demonstrated an anticancer activity by a COX2 inhibitor through both COX-dependent and -independent pathway. Previous demonstrations that NSAIDs block LNCaP cell proliferation despite low levels of COX2 protein in LNCaP cells (Hsu et al. 2000, Tanji et al. 2000, Fujita et al. 2002) also imply COX-independent anticancer effects of NSAIDs. Taken together, these results
show that NSAIDs have anticancer effects through a COX-independent pathway.

In this report, we confirmed that aspirin suppressed AR expression at a transcript level (Lim et al. 1999, Pan et al. 2003). We had speculated that aspirin regulates AR expression through PGE₂, as NSAIDs suppress PGE₂ synthesis by inhibiting COX. However, actually, PGE₂ did not affect AR mRNA or protein levels, suggesting that aspirin regulates AR expression in a COX-independent pathway. A previous report showed that a classical COX inhibitor, indomethacin, affected PG receptor expression (Chang et al. 2004). Consistently, we showed that aspirin upregulates EP3 expression, leading to an anticancer effect. Cumulatively, and in line with previous reports that NSAIDs’ anticancer effect comes through a COX-independent pathway, this study showed that aspirin regulates AR expression in a COX-independent manner by modulating EP3 expression.

We have shown, for the first time, that EP3 signaling regulates AR expression, although its mechanism is still unknown. EP3 is a G-protein-coupled receptor, and a negative regulator of cAMP, which has been shown to positively regulate AR transcription (Mizokami et al. 1994). Therefore, EP3 may negatively regulate AR expression by modulating cAMP concentration. Alternatively, NF-κB might mediate AR suppression through EP3 because EP3 signaling is known to inactivate the NF-κB pathway (Wang et al. 2010), which, in turn, positively regulates AR expression (Zhang et al. 2009). Taken together, these findings suggest that EP3 signaling negatively regulates AR expression through downstream pathways in prostate cancer cells.

In cancers other than prostate cancer, EP3 is recognized as a negative key mediator of cancer progression (Shoji et al. 2004, Fulton et al. 2006, Macias-Perez et al. 2008). Consistent with these previous findings, this study as well as another recent study showed that EP3 was suppressed in prostate cancer samples compared with normal prostate samples (Huang et al. 2013) and in castration-resistant cells compared with androgen-dependent cells. Inversely, when EP3 signaling was suppressed, LNCaP cells kept proliferating in androgen-deprived conditions, similar to castration-resistant cells. Taken together, these results imply that EP3 downregulation contributes to cancer development and progression to castration resistance through AR overexpression (Fig. 7). We have also shown that a EP3 agonist downregulated AR expression and suppressed prostate cancer cell growth, indicating that activation of EP3 signaling may offer a novel therapy against androgen-dependent prostate cancer.

Figure 7
Schematic representation of the relationship between EP3 and AR expression. (Left) NSAIDs or EP3 agonist activates EP3 signaling and suppresses AR expression, thus contributing to suppression of cancer cell proliferation. (Right) Progression from normal to prostate cancer or to castration-resistant prostate cancer; EP3 expression gradually decreases, while AR expression is upregulated. As a result, cancer proliferation is potentiated.
prostate cancer, especially when combined with androgen deprivation therapy. This is supported by previous reports that EP3 agonists suppressed cell growth in colorectal cancer (Shoji et al. 2004) and hepatocellular carcinoma (Cusimano et al. 2009) and vasculogenesis in inflammatory breast cancer (Robertson et al. 2010). COX2 selective inhibitors were expected to act as a ‘super aspirin’ that would not exert the adverse effects typical of classical NSAIDs (DeWitt 1999, Majima et al. 2003). However, in some organs such as kidneys, COX2 is expressed constitutively (Okumura et al. 2002) and is necessary for the kidney to mature after birth (Norwood et al. 2000), suggesting that even ‘super aspirin’ can cause adverse effects. Thus, selective activation of EP3 receptor signaling may be a more promising treatment than COX2 inhibition.

In conclusion, aspirin represses AR expression by upregulating EP3 expression in prostate cancer cells and exerts preventative and anticancer effects in prostate cancer by downregulating AR expression. Modulated EP3 signaling affected both AR expression and prostate cancer growth under androgen deprivation conditions. Taken together, this study showed a rationale for use of NSAID aspirin in preventing prostate cancer. We also identified a potential treatment for androgen-dependent prostate cancer in combining EP3-targeting therapy with androgen deprivation therapy.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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