Protein kinase C regulates Twist1 expression via NF-κB in prostate cancer

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Abstract

The progression of prostate cancer to metastatic and castration-resistant disease represents a critical step. We previously showed that protein kinase C (PKC) activation followed by Twist1 and androgen receptor (AR) induction played a critical role in castration resistance, but the precise molecular mechanism remains unknown. This study aimed to elucidate the relevant molecular mechanism, focusing on NF-κB transcription factor. We examined the activity of NF-κB after PKC inhibition, and the expression of Twist1 and AR after inhibition of NF-κB in human prostate cancer cells. We also investigated the status of PKC/NF-κB after inhibition of AR signaling in cells resistant to hormonal therapy. As a result, inhibition of PKC signaling using knockdown and small-molecule inhibition of PKC suppressed RelA activity, while blocking NF-κB suppressed Twist1 and AR expression. Conversely, inhibition of AR signaling by androgen depletion and the novel antiandrogen enzalutamide induced PKC and RelA activation, resulting in Twist1/AR induction at the transcript level. Moreover, inhibition of NF-κB signaling prevented enzalutamide-induced Twist1 and AR induction. Finally, NF-κB was activated in both castration-resistant and enzalutamide-resistant cells. In conclusion, NF-κB signaling was responsible for Twist1 upregulation by PKC in response to AR inhibition, resulting in aberrant activation of AR. NF-κB signaling thus appears to play a critical role in promoting both castration resistance and enzalutamide resistance in PKC/Twist1 signaling in prostate cancer.

Introduction

Androgen deprivation therapy (ADT) is currently the gold-standard treatment for recurrent or advanced prostate cancer (Sharifi et al. 2010). Most prostate cancers are initially dependent on androgen receptor (AR) signaling for cell proliferation and cellular survival, at which point most patients respond well to ADT. However, most patients with prostate cancer eventually relapse in a castration-resistant manner during ADT, which is defined as castration-resistant prostate cancer (CRPC) (Sadar 2011). Several novel AR-targeting agents against CRPC have recently been developed, including the next-generation antiandrogen enzalutamide (Ryan & Tindall 2011, Scher et al. 2012) and the cytochrome P17 inhibitor abiraterone acetate (de Bono et al. 2011, Ryan et al. 2013). However, their therapeutic effects are modest, and novel therapeutic strategies are required to improve the prognosis in men with CRPC.

The mechanisms responsible for the development of CRPC include aberrant activation of AR under low levels of circulating androgens such as de novo androgen synthesis...
Endocrine-Related Cancer

α (HIF-1) (Yang)

enzalutamide-resistant prostate cancer.

In this study, we aimed to elucidate the signaling cascade of PKC/Twist1 and to clarify their roles in castration- and

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transcription factors, such as STAT3 (Inoue & Ogawa 2011). STAT3 transcription factors may mediate PKC-induced Twist1 induction. Several transcription factors, such as STAT3 (Lo et al. 2007, Cheng et al. 2008), hypoxia-inducible factor 1α (HIF-1α) (Yang et al. 2008) and NF-κB (Pham et al. 2007), have been reported to regulate Twist1 expression directly. In this study, we aimed to elucidate the signaling cascade of PKC/Twist1 and to clarify their roles in castration- and enzalutamide-resistant prostate cancer.

Materials and methods

Cell culture

Human prostate cancer LNCaP, C4-2 and 22Rv1 cells were cultured in RPMI-1640 (Invitrogen) containing 10% fetal bovine serum. LNCaP and 22Rv1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and authenticated by short tandem repeat (STR) analysis. LNCaP cells were used after 10–40 rounds of propagation. C4-2 cells authenticated by whole-genome and whole-transcriptome sequencing on an Illumina Genome Analyzer IIX platform (Lamoureux et al. 2011) were kindly provided by Dr Martin Gleave (Vancouver Prostate Centre, Vancouver, BC, Canada). Castration-resistant derivatives of LNCaP cells (LNCaP-CxR cells, referred to as CxR cells) were established and maintained as described previously (Shiota et al. 2010). Enzalutamide-resistant derivatives of LNCaP and C4-2 cells (LNCaP/MDV and C4-2/MDV cells) were established by long-term culture in the appropriate media containing gradually increasing concentrations of enzalutamide and maintained in media containing 50μM enzalutamide (Shiota et al. 2014b). The cell lines were maintained in a 5% CO2 atmosphere at 37°C.

Antibodies and reagents

Antibodies against RelA (sc-372), AR (N-20, sc-816) and Twist1 (sc-81417) were purchased from Santa Cruz Biotechnology. Antibodies against phosphorylated RelA Ser536 (p-RelA, #3033), phosphorylated PKC Ser660 (p-PKC; #9371), phosphorylated STAT3 Ser727 (p-STAT3Ser727; #9134) and STAT3 (#4904) were purchased from Cell Signaling Technology. Anti-PKC (SAB4502356), HIF-1α (HPA001275) and anti-β-actin (A3854) antibodies were obtained from Sigma-Aldrich.

Transfection with small-interfering RNAs

Double-stranded small-interfering RNA (siRNA) 25 base pair oligonucleotides were generated commercially (Invitrogen). The target sequences are listed in Supplementary Table 1 (see section on supplementary data given at the end of this article). Prostate cancer cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

RNA isolation, reverse transcription and quantitative real-time PCR

RNA isolation and reverse transcription were performed as described previously (Shiota et al. 2014a, 2016). Quantitative real-time PCR was performed using TaqMan Gene Expression Assays for RelA (Hs00153294_m1), Twist1 (Hs00361186_m1), full-length AR (Hs00171172_m1), AR V7 (made to order), HIF-1α (Hs00153153_m1), KLK3 (Hs00426859_g1), FBIP5 (Hs01561006_m1), PKRCE (Hs00942877_m1) and the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH; Hs02758991_g1) (Applied Biosystems) with TaqMan Gene Expression Master Mix (Applied Biosystems) with a 7900HT PCR system (Applied Biosystems). The transcript levels of the target genes were corrected according to the corresponding GAPDH transcript levels. All values represent the results of at least three independent experiments.
**Western blotting analysis**

Whole-cell extracts were prepared as described previously (Shiota et al. 2014a, 2016). Briefly, the concentrations of the prepared protein extracts were quantified using a protein assay (Bio-Rad) based on the Bradford method. Aliquots (30 μg protein) were separated by 4–20% SDS-PAGE and transferred to polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science) using a semi-dry blotter. The membranes were then incubated with the primary antibodies described previously for 1 h at room temperature, followed by incubation with peroxidase-conjugated secondary antibodies for 40 min at room temperature. The bound antibodies were visualized using an ECL kit (GE Healthcare Bio-Science), and images were obtained using an image analyzer (Ez-Capture MG, ATTO, Tokyo, Japan).

**Luciferase reporter assay**

Prostate cancer cells (2 × 10⁵) were co-transfected with 0.5 μg of NF-κB reporter plasmid (pGL4.32(luc2P/NF-κB-RE/Hygro), Promega), and 0.05 μg of pRL-TK as an internal control with or without 40 nM of the indicated siRNAs using Lipofectamine 2000. After 48 h, the luciferase activities were detected using a Dual-Luciferase Reporter Assay System (Promega). The light intensities were measured using the ARVO MX plate reader. The firefly luciferase activities were corrected by the corresponding Renilla luciferase activities. The results are representative of at least three independent experiments.

**Statistical analysis**

All data were assessed using the Student t-test. All P values are two sided. Levels of statistical significance were set at P < 0.05.

**Results**

**Blocking PKC signaling suppresses RelA activity**

To explore the transcription factor responsible for Twist1 expression induced by PKC activation, we examined the known regulators of Twist1 transcription STAT3, HIF-1α and NF-κB. PKCε regulating Twist1 expression (Shiota et al. 2014a) were silenced in LNCaP and 22Rv1 cells by specific siRNAs and checked phosphorylated STAT3 levels. However, PKC knockdown did not prominently suppress STAT3 phosphorylation in LNCaP and 22Rv1 cells (Supplementary Fig. 1A), whereas the PKC inhibitor Ro31-8220 suppressed phosphorylated STAT3 in 22Rv1 cells, but not in LNCaP cells (Supplementary Fig. 1B). Similarly, we investigated the involvement of HIF-1α. PKC inhibition using siRNA significantly reduced mRNA levels of their target genes (Supplementary Fig. 2A) and marginally decreased HIF-1α mRNA and protein expression in both LNCaP and 22Rv1 cells (Supplementary Fig. 2B and C), whereas the PKC inhibitor Ro31-8220 significantly suppressed HIF-1α at both mRNA and protein levels (Supplementary Fig. 2D and E). However, HIF-1α knockdown did not suppress Twist1 or AR expression in LNCaP and 22Rv1 cells (Supplementary Fig. 3A and B). Taken together, these inconsistent results suggested that STAT3 and HIF-1α were not robustly involved in Twist1/AR signaling as a downstream of PKC, at least in both LNCaP and 22Rv1 cells although there remains the possibility that these molecules may partially play a role in this pathway.

We subsequently examined the role of NF-κB in PKC/Twist1 signaling to find out the robust molecule. The PKC inhibitor Ro31-8220 suppressed RelA expression at the transcriptional level in both LNCaP and 22Rv1 cells (Fig. 1A). Furthermore, Ro31-8220 suppressed the phosphorylation and protein expression of RelA in both LNCaP and 22Rv1 cells (Fig. 1B). We used siRNAs specific for PKCε to confirm that PKC regulates RelA. Although PKC knockdown did not affect RelA expression at the transcriptional level (Fig. 1C), PKCε knockdown suppressed RelA phosphorylation in LNCaP and 22Rv1 cells (Fig. 1D). In addition, reporter assay measuring NF-κB activity showed consistent results that the PKC inhibitor and PKC knockdown suppressed NF-κB activities (Fig. 1E).

**NF-κB regulates Twist1/AR signaling**

We investigated the effect of NF-κB on Twist1/AR signaling as a downstream of PKC by inhibiting RelA activation directly. When two kinds of RelA-specific siRNAs were utilized, RelA knockdown suppressed Twist1 and AR mRNA expression in LNCaP cells and Twist1, full-length AR and V7 mRNA expression in 22Rv1 cells (Fig. 2A). RelA knockdown consistently suppressed Twist1 and full-length AR expression at the protein level in LNCaP cells, whereas expression levels of Twist1, full-length AR and AR variants were affected by RelA knockdown in 22Rv1 cells, especially when RelA siRNA #2 was utilized (Fig. 2B).
Blocking AR signaling induces PKC/RelA activation, resulting in Twist1/AR activation

Given the previously mentioned results, we examined the effects of androgen depletion on PKC/RelA and Twist1/AR signaling. RelA mRNA expression was increased in LNCaP cells subjected to androgen depletion, but not in 22Rv1 cells (Fig. 3A). In addition, RelA phosphorylation was augmented by androgen depletion in LNCaP cells (Fig. 3B). We previously showed that PKC phosphorylation was augmented by androgen depletion, leading to the upregulation of Twist1 and AR expression (Shiota et al. 2014a). Similarly, phosphorylation of PKC and RelA was increased by androgen depletion in 22Rv1 cells (Fig. 3B). Consistently, Twist1, full-length AR and AR V7 mRNA expression levels were increased by androgen deprivation in 22Rv1 cells (Fig. 3C). Furthermore, Twist1 and AR variant protein expression levels were markedly augmented by androgen depletion, whereas full-length AR protein expression was less augmented, possibly because of...
increased protein degradation by lack of ligand binding under androgen depletion (Fig. 3D).

We also blocked AR signaling using enzalutamide, effects of which were confirmed by the suppression of AR target genes KLK3 and FKBP5 mRNA levels with enzalutamide (Supplementary Fig. 4B). Twist1 and AR mRNA expression levels in LNCaP cells were increased by enzalutamide (Shiota et al. 2014a), whereas RelA mRNA expression was unaffected (Fig. 4A). Similarly, Twist1, full-length AR and AR V7 mRNA levels in 22Rv1 cells were increased by enzalutamide, whereas RelA mRNA was not (Fig. 4B). RelA phosphorylation was also augmented in LNCaP cells (Fig. 4C), consistent with increased phosphorylation of PKC and increased Twist1 and AR expression, as previously shown (Shiota et al. 2014a). PKC phosphorylation and protein expression levels of RelA, Twist1 and AR variants were augmented by enzalutamide in 22Rv1 cells, whereas full-length AR protein expression was less augmented, possibly because of increased protein degradation by blocking of ligand binding by enzalutamide (Fig. 4D).

**Inhibition of NF-κB signaling prevents enzalutamide-induced Twist1 and AR expressions**

To delineate the role of NF-κB signaling in Twist1 and AR expression induced by blocking AR signaling, we examined Twist1 and AR expression levels after enzalutamide treatment, with or without blocking NF-κB signaling, in castration-resistant 22Rv1 cells. RelA knockdown with enzalutamide suppressed RelA, Twist1 and full-length AR mRNA expression levels in 22Rv1 cells, whereas AR V7 was little affected by RelA knockdown (Fig. 5A). This may be because AR V7 expression was induced by complementary pathways, such as the RSK/YB-1 pathway (Shiota et al. 2016). Similarly, RelA, Twist1 and full-length AR protein expression levels were reduced by RelA knockdown, whereas AR variant protein expression was little affected (Fig. 5B).

**NF-κB is activated in CRPC and enzalutamide-resistant cells**

To reveal the biological relevance of NF-κB signaling, we examined the activation status of NF-κB signaling in CRPC and enzalutamide-resistant prostate cancer cells. RelA mRNA expression and levels of phosphorylated RelA were augmented in castration-resistant CxR cells (Fig. 6A and B), as well as NF-κB activity indicated by reporter assay in castration-resistant 22Rv1 cells, compared with LNCaP cells (Supplementary Fig. 5A). Similarly, RelA mRNA expression and phosphorylated RelA were augmented in enzalutamide-resistant LNCaP/MDV and C4-2/MDV cells (Fig. 6C and D), where PKC activities appeared to be...
augmented in enzalutamide-resistant cells (Supplementary Fig. 5B). This finding was partially confirmed by reporter assay indicating higher NF-κB activity in C4-2/MDV cells compared with that in C4-2 cells (Fig. 6E).

**Discussion**

Previous studies showed that PKC/Twist1 signaling induced by AR-targeting therapy resulted in the development of CRPC via augmented AR expression (Shiota et al. 2010, 2014a). However, the mechanism of Twist1 induction by activated PKC remains unclear. In this study, we identified a role for the transcription factor NF-κB in Twist1 upregulation by activated PKC, whereas the other known Twist1 regulators STAT3 or HIF-1α were not involved. In addition, NF-κB has previously been shown to regulate AR expression directly (Zhang et al. 2009), suggesting that NF-κB thus regulates AR expression directly as downstream of PKC.

RelA phosphorylation by various kinases at each phosphorylation site is required for optimal induction of NF-κB target genes in response to distinct stimuli (Viatour et al. 2005). RelA Ser536 may be phosphorylated by a variety of kinases, such as IkB kinases, TANK-binding kinase, Akt and ribosomal s6 kinase, via various signaling pathways (Viatour et al. 2005). However, no studies have demonstrated direct phosphorylation of RelA at Ser536 yet, though PKC has been shown to phosphorylate RelA at Ser311 (Duran et al. 2003). The results of the current study thus suggest that phosphorylation of RelA at Ser536...
may be affected directly or indirectly via PKCs. NF-κB was previously reported to be activated in response to androgen depletion in an LNCaP xenograft model (Chiu et al. 2010). Similarly, AR was shown to be a negative regulator of NF-κB transcriptional activity (Palvimo et al. 1996, Cinar et al. 2004). Although the mechanism of NF-κB activation remains unclear, the present study consistently showed that NF-κB as well as enzalutamide was activated

Figure 4
Enzalutamide induces PKC/RelA activation and Twist1/AR suppression. (A) and (B) LNCaP (A) and 22Rv1 (B) cells were incubated with 10 μM enzalutamide for the indicated duration. Quantitative real-time PCR was performed using the indicated primers and probes. The expression level of the target transcript was corrected according to the corresponding GAPDH transcript level. All values represent the results of at least three independent experiments. The level of each transcript in cells treated for 0 h was defined as 1. Boxes: mean; bars: ± s.d. *P < 0.05 (compared with 0 h). (C) and (D) LNCaP (C) and 22Rv1 (D) cells were incubated with 10 μM enzalutamide for the indicated duration. Whole-cell extracts were subjected to SDS-PAGE, followed by Western blotting analyses of the indicated proteins.

Figure 5
Inhibition of NF-κB signaling prevents enzalutamide-induced Twist1 and AR expressions. (A) 22Rv1 cells were transfected with 40 nM of control siRNA or RelA siRNA #2, incubated for 24 h, and were further incubated with 10 μM enzalutamide for 24 h. Quantitative real-time PCR was performed using the indicated primers and probes. The expression level of the target transcript was corrected according to the corresponding GAPDH transcript level. All values represent the results of at least three independent experiments. The level of each transcript in mock-transfected cells was defined as 1. Boxes: mean; bars: ± s.d. *P < 0.05 (compared with control siRNA). (B) 22Rv1 cells were transfected with 40 nM of control siRNA or RelA siRNA #2, incubated for 24 h, and were further incubated with 10 μM enzalutamide for 48 h. Whole-cell extracts were subjected to SDS–PAGE, followed by Western blotting analyses of the indicated proteins.
NF-κB is activated in CRPC and in enzalutamide-resistant cells. (A) After extraction of total RNA from LNCaP and CxR cells and synthesis of cDNA, quantitative real-time PCR was performed using the indicated primers and probes. The expression level of the target transcript was corrected according to the corresponding GAPDH transcript level. All values represent the results of at least three independent experiments. The level of each transcript in LNCaP cells was defined as 1. Boxes: mean; bars: ±s.d. *P < 0.05 (compared with LNCaP cells). (B) Whole-cell extracts from LNCaP and CxR cells were subjected to SDS–PAGE, followed by Western blotting analyses of the indicated proteins. (C) After extraction of total RNA from LNCaP, LNCaP/MDV, C4-2 and C4-2/MDV cells and synthesis of cDNA, quantitative real-time PCR was performed using the indicated primers and probes. The expression level of the target transcript was corrected according to the corresponding GAPDH transcript level. All values represent the results of at least three independent experiments. The level of each transcript in LNCaP cells was defined as 1. Boxes: mean; bars: ±s.d. *P < 0.05 (compared with LNCaP cells). **P < 0.05 (compared with C4-2 cells). (D) Whole-cell extracts from LNCaP, LNCaP/MDV, C4-2 and C4-2/MDV cells were subjected to SDS–PAGE, followed by Western blotting analyses of the indicated proteins. (E) LNCaP, LNCaP/MDV, C4-2 and C4-2/MDV cells were co-transfected with 0.5 μg of NF-κB reporter plasmid and 0.05 μg of pRL-TK. All values are representative of at least three independent experiments. The luciferase activity of the NF-κB reporter plasmid in LNCaP and C4-2 cells was set as 1. Boxes: mean; bars: ±s.d. *P < 0.05 (compared with LNCaP and C4-2 cells).

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0384.
Declaration of interest
Masaki Shioita, Akira Yokomizo and Masatoshi Eto have received honoraria from Astellas Pharma.

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