Suppression of the androgen receptor function by quercetin through protein–protein interactions of Sp1, c-Jun, and the androgen receptor in human prostate cancer cells

Huiqing Yuan · Charles Y. F. Young · Yuanyuan Tian · Zhifang Liu · Mengye Zhang · Hongxiang Lou

Abstract We have previously reported that the increase in c-Jun expression induced by quercetin inhibited androgen receptor (AR) transactivation, and Sp1 was involved in quercetin-mediated downregulation of AR activity. Transient transfection assays in this work revealed that co-expression of c-Jun quenched Sp1-induced production of luciferase activity driven by AR promoter or three copies of Sp1 binding elements in the AR promoter. Moreover, c-Jun repressed AR-mediated luciferase activity via androgen-response elements (AREs) of the hK2 gene, while this suppression could be restored partially by cotransfection of Sp1 expression plasmid. The physical associations of c-Jun, Sp1, and AR induced by quercetin were further demonstrated by co-immunoprecipitation experiments. In addition, quercetin-mediated repression of AR expression and activity was partially reversed by blocking of JNK signaling pathway. These results suggested that c-Jun might play an important role in the suppression of AR expression and activity in the presence of quercetin, and association of a c-Jun/Sp1/AR protein complex induced by quercetin represented a novel mechanism that was involved in down-regulation of the AR function in prostate cancer cells.

Abbreviations

AR	Androgen receptor
ARE	Androgen-response element
AP-1	Activator protein-1
hK2	Human glandular kallikrein
Mib	Mibolerone
PSA	Prostate-specific antigen
Sp1	Promoter specificity protein 1

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Introduction

The human androgen receptor (AR), as a member of the superfamily of nuclear receptors, is a ligand-dependent transcription factor that mediates the biological actions of androgens [1, 2]. Many independent lines of evidence indicate that the AR is a pivotal molecule in normal development of the prostate and in the development and progression of prostate cancer [3–5]. Currently, AR is the main target for treatment of advanced prostate cancer. Proper regulation of the AR action may be able to reduce development and progression of this cancer.

The transcriptional activity of AR is modulated by a number of coregulators including c-AMP response-element-binding protein (CREB) binding protein (CBP)/p300, steroid receptor coactivators (SRC-1), and others [5–7]. In addition, functional and physical interactions between AR

and other transcription factors such as Smad3 [8] and AP1/c-Jun [9] result in transrepression of the AR function, whereas associations with members of the Ets family [10] and Heat-Shock Protein 27 [11] enhance the AR function in an androgen dependent way. The ubiquitous transcription factor Sp1 has been shown to be associated with some sequence-specific transcription factors such as NF-κB [12], Oct-1 [13], and synergistically activate the expression of target genes. Moreover, several reports have demonstrated that Sp1 interacts with estrogen receptor (ER) [14], progesterone receptor (PR) [15], and AR [16, 17]. Studies of Curtin et al. [16] demonstrate that androgen suppresses the expression of GnRH-stimulated rat LHß gene. The inhibition is through protein–protein interaction between AR and Sp1 that reduces Sp1 DNA binding activity in clonal gonadotrope cell line.

c-Jun protein has been extensively characterized as a main component of AP-1 protein complex which can directly activate or induce transcription of many genes containing AP-1 DNA binding sequences. It is known that a functional interaction between c-Jun and AR is responsible for either a positive or a negative effect on the AR-mediated transcriptional activity. The studies of Bubulya et al. [18] and Wise et al. [19] provide evidence that the c-Jun potentiates the AR transcription activity by enhancing both the AR homodimerization and DNA binding activity in COS cells. In contrast, the data from Sato et al. [9] and Lobaccaro et al. [20] demonstrate that a direct protein–protein interaction of AR/c-Jun results in inhibition of AR function in prostate cancer cells.

We previously showed that the significant induction of c-Jun was observed in prostate cancer cells treated with quercetin, which suppressed the AR function [21, 22]. Subsequent investigation confirmed that Sp1 served as a coactivator to facilitate the activity of AR [23]. In this study, we went a step further to provide evidence that a protein complex containing c-Jun, Sp1, and the AR was formed in response to quercetin treatment and mediated the suppression of AR function.

Materials and methods

Cell culture and chemicals

Human prostate cancer cell lines LNCaP (The American Type Culture Collection, Rochville, MD, USA) and PC-3 (The Cell Bank of Chinese Academy of Sciences, Shanghai) were seeded in 100 mm dishes, 75 ml flasks, or 12-well plate culture dishes in RPMI 1640 medium (Celox, St Paul, MN, USA or HyClone) supplemented with 5% fetal bovine serum (FBS) (Biofluids, Rockville, MD, USA or JRH biosciences, Australia) and 5% CO_2 at 37°C until

reaching approximately 60–70% confluence. Cells were maintained in serum-free RPIM 1640 medium for further 24 h to deplete endogenous steroid hormones before experiments, and then treated with quercetin (100 μM) with or without 1 nM of mibolerone (Mib) in the medium containing 1% charcoal-stripped serum. Mibolerone (New England Nuclear, or HyClone) was dissolved in ethanol. Quercetin (LKT, Inc. St. Paul, MN, USA) was dissolved in DMSO, which was also used as a control vehicle.

Transient transfection and the reporter gene assay

Cells were seeded in 12-well plates at a density of 0.6×10^5/well, and grown under the conditions described above. For transfection into LNCaP cells, either luciferase reporter plasmid with 6 kb PSA promoter (PSA promoter, 1 μg/well), or pGL3 basic vector-containing AR2 kb promoter (AR promoter, 1 μg/well), or pGL3-SV40 construct with three copies of ARE elements in hk2 gene (pGL3-SV40-hk2-3ARE, 1 μg/well), or pGL3-SV40 with three copies of Sp1 binding motif in the AR promoter (pGL3-SV40-AR-3Sp1, 1 μg/well) [24, 25], and pcDNA-Sp1 expressing Sp1 and/or pRSV-c-Jun expression vector as indicated were co-transfected using GeneFECTOR™ Transfection Reagent (Venn Nova, LLC). For transfection into PC-3 cells, human AR expression vector pSG5-AR (0.5 μg/well) was co-transfected with desired plasmids using Lipofectamine2000 (Invitrogen Life Technologies). The parental vectors pGL3 basic (1 μg/well) and pGL3-SV40 (1 μg/well) were used as controls. An expression vector containing a CMV promoter/β galactosidase (pCMV-β-gal, 0.2 μg/well) was co-transfected as an internal control to normalize transfection efficiency. After 24 h of transfection, cells were either treated with quercetin or with addition of inhibitor SP600125 (10 μM) for 30 min, followed by administration of quercetin in the presence or absence of Mib (1 nM) for an additional 24 h in the medium with 1% charcoal-stripped serum. Cells remained untreated served as a control. The cell lysates were used for β-gal assay and luciferase assays (Promega, Madison, WI, USA). At least three independent transfections were performed.

Protein preparation and western blot analysis

Cells were plated in 100-mm dishes under the same conditions described above, and treated with chemicals as indicated or remained untreated in the presence or absence of 1 nM Mib for 24 h. After treatment, cells were washed twice with cold phosphate buffered saline (PBS), and cell pellets were obtained by centrifugation. Whole cell lysates were prepared according to the method described previously [23, 26]. Freshly prepared protease inhibitors (0.5 mM

phenylmethanesulfonyl fluoride (PMSF), 50 µg/ml aprotinin, 10 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM β-glycerolphosphate) were also added. The Bradford protein assay (Bio-Rad, Hercules, CA, USA) was employed for quantifying the protein content. Proteins were resolved on a SDS-polyacrylamide gel (4–12%), and separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The blots were then blocked in TBST buffer (20 mM Tris–HCl, 137 mM NaCl, and 0.1% tween 20, pH 8.0) containing 5% non-fat milk prior to incubation with specific antibodies to Sp1 (Santa Cruze Biotechnology Inc.), phos-c-Jun (Cell Signaling Technology), AR (BD Biosciences) or β-tubulin (Santa Cruz Biotechnology) overnight at 4°C. Membranes were incubated with an anti-rabbit or anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech UK limited, and Santa Cruz Biotechnology) at room temperature for 1 h and visualized by enhanced chemiluminescence substrate (ECL, Amersham Corporation). For detection of AR protein produced by the AR-expressing plasmid in transfected cells, we transfected the human AR expression vector pSG5-AR into PC-3 cells, and cotransfected pGL3-SV40 reporter or pGL3-hk2-3ARE construct in cells grown in 75 ml flasks. After 24 h transfection, the cells were treated with chemicals as indicated for an additional 24 h, and then the cell lysates were prepared for western blot assay. Densitometric analysis for AR and phosphor-c-Jun protein levels normalized to β-tubulin using Gel-Proanalyzer 4(Media Cybernetics).

Co-immunoprecipitation

LNCaP cells were cultured with or without Mib or Mib combined with quercetin for 24 h in medium containing 1% charcoal stripped serum. Proteins in whole cell lysates were precleared with anti-rabbit IgG or anti-mouse IgG and protein A-agarose (Santa Cruz Biotechnology, Inc.). Aliquots of 500 µg precleared proteins were incubated with 2 µg of antibodies directed against Sp1 (Santa Cruz Biotechnology, Inc.), c-Jun (Cell Signaling Technology) or AR (BD Biosciences) in binding buffer (20 mM HEPES, pH7.9, 20% glycerol, 150 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 50 µg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM β-glycerolphosphate) overnight at 4°C. The protein A beads were added and incubated for a further 2 h at 4°C. The immunoprecipitates were washed four times with buffer containing 50 mM Tris–HCl, pH7.5, 0.5% IGEPAL CA-630, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 50 µg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM β-glycerolphosphate. Immunocomplexes were recovered by heating at 75°C for 10 min in SDS sample buffer and analyzed by electrophoresis using 7% or 12% NuPAGE Tris–acetate gel (invitrogen). Western blot detection was performed as described above.

Statistical analysis

The data are presented as the mean ± SD of several independent experiments. Statistical analysis was performed using two-tailed Student's t test for paired values, and statistical significance was considered when P was <0.05.

Results

Overexpression of c-Jun inhibited Sp1-mediated induction of AR gene

We previously reported that quercetin had inhibitory effect on AR gene transcription and function, and the induction of c-Jun resulted in suppression of AR expression and activity [21, 22]. Since Sp1 is a major regulator of the AR expression [27], and Sp1 was involved in quercetin-mediated inhibition of AR expression and activity [23]. It remains unclear whether induction of c-Jun by quercetin is linked directly to Sp1, and subsequently affects AR gene expression. Therefore, we tested this possibility by performing cotransfection assays with expression vector encoding either c-Jun or Sp1 into LNCaP cells, and luciferase reporter activity of the AR promoter was measured. As shown in Fig. 1A, expression of c-Jun inhibited activity of the AR promoter, and Sp1 was able to stimulate the AR promoter activity, while cotransfection of c-Jun attenuated the Sp1-mediated stimulation on AR promoter activity. To determine if this c-Jun effect is mediated through Sp1, a vector only containing three copies of tandem Sp1 binding elements of the AR promoter fused to luciferase cDNA was used as a reporter for this purpose. As expected, Fig. 1B showed that the negative effect of c-Jun was observed via the Sp1 binding site in the AR promoter. These results suggested that the inhibitory effect of c-Jun on AR expression might be caused by association with Sp1 protein.

Overexpression of c-Jun mediated functional interference on the transactivity of AR

We previously showed that quercetin could induce expression of c-Jun which in turn inhibited AR's transcriptional activity [22]. It has been reported that there is a direct protein–protein interaction between the AR and c-Jun that might affect the function of AR [9]. We reasoned that if c-Jun attenuated the function of AR, then the
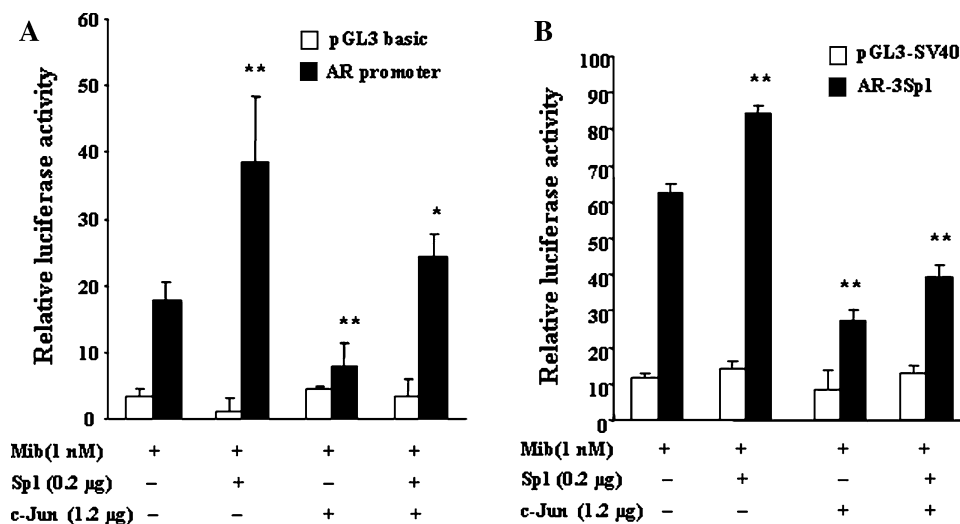
Fig. 1 Sp1-mediated stimulation of the AR promoter activity was suppressed by co-expression of c-Jun. **A** LNCaP cells were co-transfected with luciferase reporter of AR promoter. $* P < 0.05$, compared with group AR promoter plus Sp1 expression plasmid; $** P < 0.01$, when compared with group AR promoter plus Mib treatment. **B** LNCaP cells were co-transfected with pGL3-SV40 with three copies of Sp1 elements of the AR gene (AR-3Sp1) luciferase reporter, together with Sp1 and c-Jun expression vectors in the indicated combinations. The parental vectors of pGL3 basic and pGL3-SV40 were included as controls. In each transfection, the pCMV-β-galatosidase (β-gal) was co-transfected to serve as an internal control for transfection efficiency. The LNCaP cells were incubated with 1 nM of Mib for 24 h after transfection. The normalized, relative luciferase activities (mean $\pm$ SE) of at least three independent experiments performed in duplicate were shown. $** P < 0.01$ when compared with group AR-3Sp1 plus Mib treatment, or with AR-3Sp1 + Sp1

induction of c-Jun by quercetin should have a functional interaction with the AR. The human glandular kallikrein-2 (hk2) is androgen-inducible gene that contains androgen-response elements (AREs) to which AR binds. Expression of this gene is highly dependent on the transactivity of AR. To determine the effect of c-Jun on AR's function, cotransfection assays were first performed with expression vector encoding c-Jun and luciferase reporter construct containing three tandem repeats of ARE element of the hk2 gene in LNCaP cells, AR activity was determined by measuring the level of luciferase-reporter gene activity following transfection. As shown in Fig. 2A, luciferase activity decreased when c-Jun was cotransfected into cells in the presence of androgen, and expression of c-Jun exerted inhibitory effect on AR activity in a dose-dependent manner. Since the luciferase reporter construct hk2-3ARE-Luc only contains three copies of ARE element, the decrease of luciferase activity mediated by c-Jun might be a result from the specifically functional interaction of c-Jun and the AR. We further evaluated the importance of this functional association of c-Jun and AR using PC3 cell line, which lacks endogenous AR. As expected, androgen induced production of luciferase of hk2-3ARE-Luc reporter was blunted with co-expression of c-Jun and AR (Fig. 2B), which were consistent with result shown in Fig. 2A. The AR protein levels in PC-3 cells transfected with AR-expressing plasmid were analyzed and shown in Fig. 2C. Together, these data suggested that functional interaction of c-Jun and AR resulted in the inhibition of the AR activity.

Functional interactions of c-Jun, Sp1, and the AR blocked the transactivity of AR

The above results led us to test whether c-Jun and Sp1 are involved in downregulation of AR activity in prostate cancer cells. As shown in Fig. 3A, activity of the luciferase reporter plasmid containing the PSA6 kb promoter was examined by cotransfection into LNCaP cells with a constant amount of c-Jun expression vector and with increasing amounts of Sp1 expression plasmid as indicated. Indeed, c-Jun dramatically repressed the PSA-promoter activity, while this suppression was restored partially by co-transfection of the Sp1 expression vector in a dose-dependent fashion. Reciprocally, luciferase production of hk2-3ARE-Luc was analyzed by cotransfection of Sp1 expression alone or combined with indicated amounts of c-Jun expression plasmid. As expected, the results in Fig. 3B showed that the enhancement of the AR activity by Sp1 was repressed by coexpression of c-Jun, cotransfection of higher amount of c-Jun expression vector resulted in a stronger repression of hk2-3ARE luciferase reporter activity. Finally, the PC-3 cell line was used to confirm the interference effect of c-Jun specifically on the cooperativity of Sp1 and the AR. The results were shown in Fig. 3C and D, which were consistent with that observed in Fig. 3A and

Fig. 2 Transactivity of the AR was impaired by overexpression of c-Jun. **A** LNCaP cells were co-transfected with luciferase reporter plasmid pGL3-SV-40 with three copies of ARE of the hk2 gene (hk2-3ARE) and varied amounts of c-Jun expression vector as indicated. *** $P < 0.001$, when compared with untreated control, or with group hk2-3ARE plus Mib treatment. ** $P < 0.01$, when compared group hk2-3ARE plus Mib treatment. **B** PC3 cells were cotransfected with pGL3-SV40-hk2-3ARE (hk2-3ARE) luciferase reporter, the indicated amount of c-Jun expression plasmid and the human AR expression vector pSG5-AR (hAR). The pCMV-β-galatosidase serves as an internal control to monitor transfection efficiency. The parental vector pGL3-SV40 was also included as a control. The normalized, relative luciferase activities (mean ± SE) of at least three independent experiments were shown. * $P < 0.05$, when compared with untreated control. ** $P < 0.01$ versus hk2-3ARE plus Mib treatment. **C** Western blot analysis of AR protein levels in whole cell lysates from PC-3 cells transfected with AR-expressing plasmid and then treated with Mib. Beta-tubulin was served as an internal control. Densitometric measurements were used for analysis of AR protein levels normalized to β-tubulin
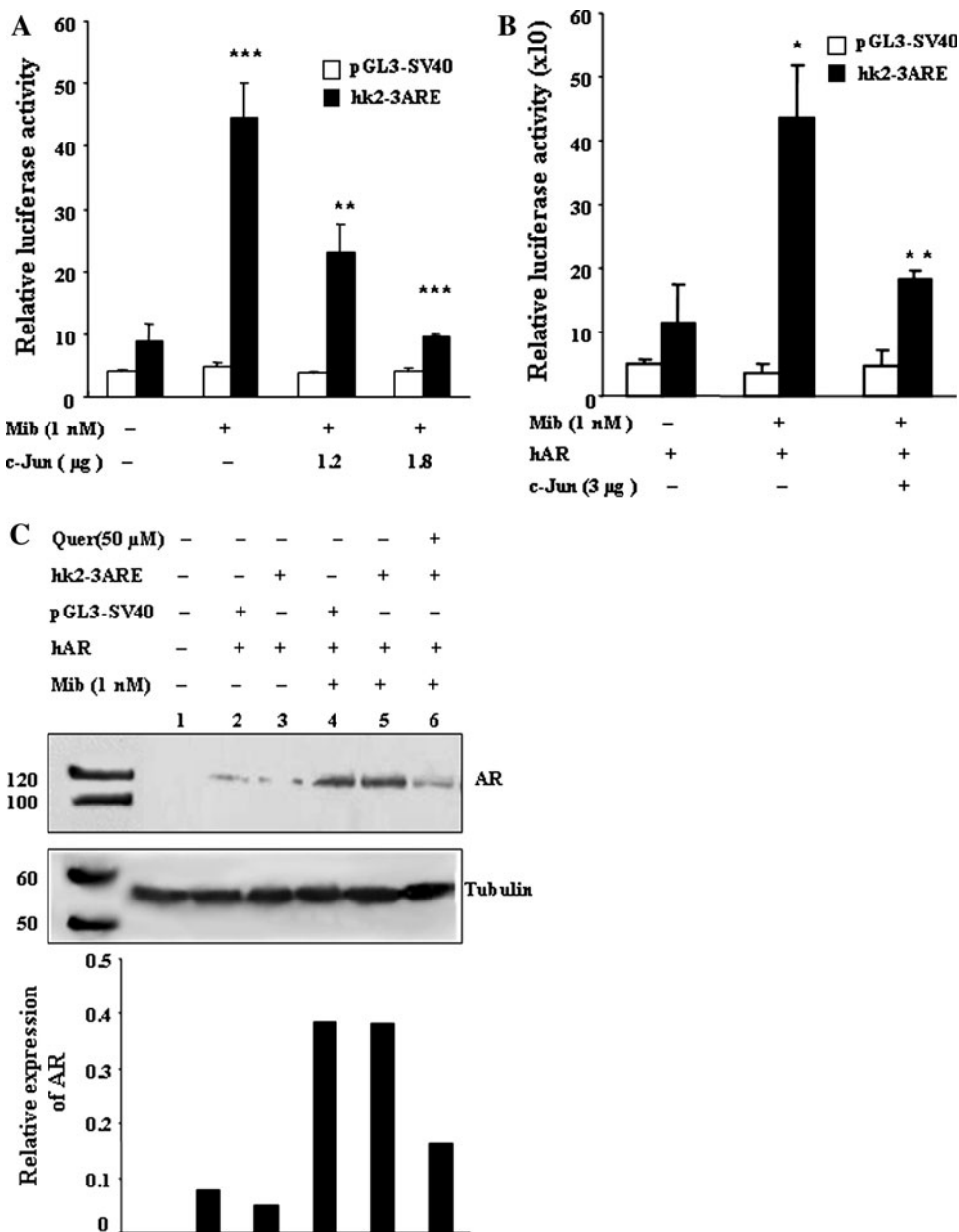

B. Therefore, the results showed that increase in c-Jun protein level had a dominant inhibitory effect over Sp1's enhancing function on androgen-regulated reporter activities. The functional associations of these three proteins seemed to be necessary for the inhibition of AR transactivity.

Physical interactions of c-Jun and the AR were induced by quercetin in vivo

The functional interactions of c-Jun, Sp1, and the AR showed above suggested that there might be physical interactions among these three factors in the presence of quercetin. It has been demonstrated that association of AR and Sp1 resulted in the suppression of AR activity in response to quercetin treatment [23]. We subsequently tested the possibility that interactions of c-Jun, Sp1, and the AR were induced by quercetin by coimmunoprecipitation and western blotting analysis. Proteins immunoprecipitated by AR antibody were subsequently subjected to western blot analyses with anti-AR, or anti-c-Jun antibody. The top panel in Fig. 4A showed AR expression in input and in co-precipitated complex as a control. The lower panel was the c-Jun content of the various immunoprecipitates. It showed that c-Jun was predominantly detected in immunoprecipitate from cells treated with quercetin in the presence of Mib (lane 6 from left in Fig. 4A), while no detectable c-Jun was seen in precipitates from cells with no
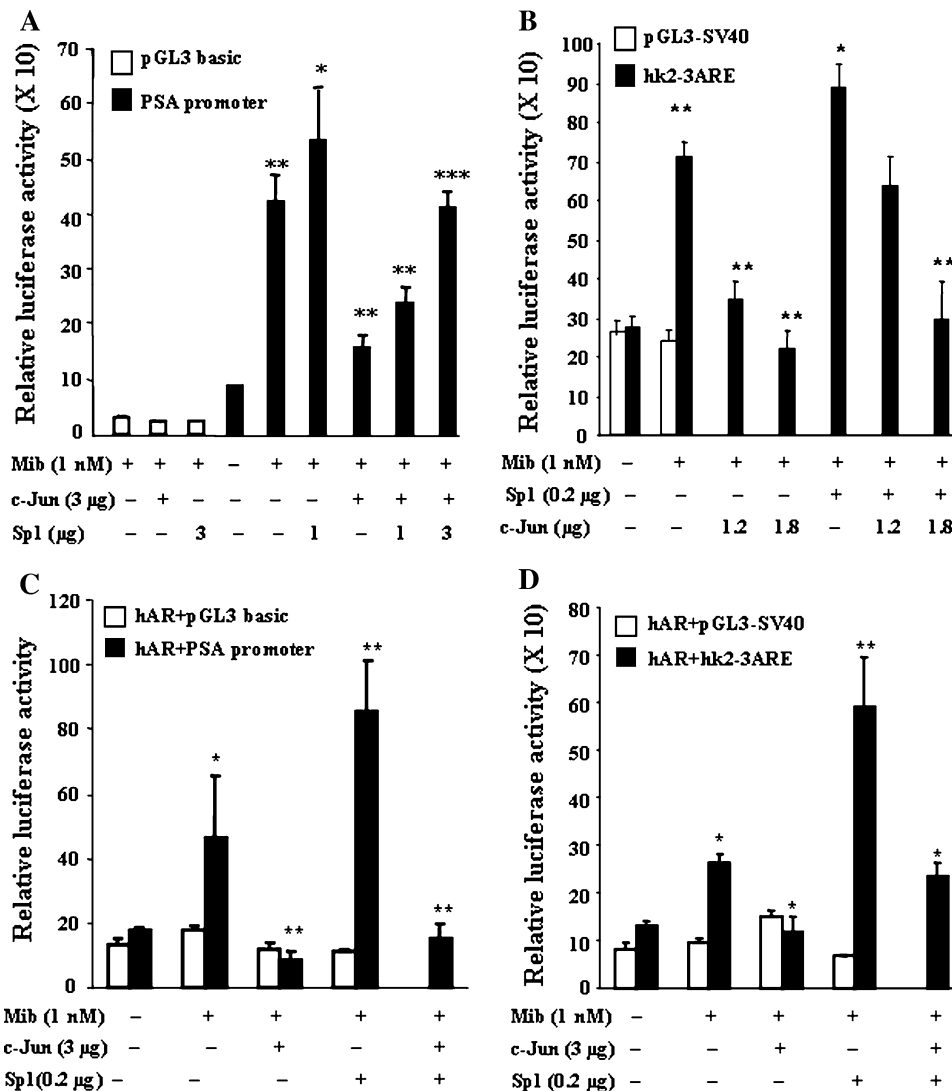
Fig. 3 Sp1-mediated stimulation on the transcription activity of the AR was relieved by cotransfection of c-Jun. **A** LNCaP cells were cotransfected with pGL3-PSA6 kb promoter (PSA promoter) luciferase reporter, Sp1, and c-Jun expression vectors as indicated. * $P < 0.05$, compared with group of Mib treatment, ** $P < 0.01$, compared with untreated control, or with Mib treatment, or PSA promoter plus c-Jun. ***$P < 0.001$, when compared with the PSA promoter plus c-Jun. **B** LNCaP cells were cotransfected with pGL3-SV40-hk2-3ARE (hk2-3ARE) luciferase reporter, Sp1, and c-Jun expression vectors as indicated. * $P < 0.05$, compared with group of Mib treatment; ** $P < 0.01$, compared with untreated control, or with Mib treatment, or hk2-3ARE plus Sp1. **C** PC3 cells were co-transfected with luciferase reporter of pGL3-PSA6 kb promoter (PSA promoter), human AR expression construct (hAR) and different amounts of Sp1 and c-Jun expression vectors in the indicated

combinations. * $P < 0.05$, compared with untreated control; **$P < 0.01$, compared with Mib treatment, or with PSA promoter plus Sp1. **D** PC3 cells were co-transfected with luciferase reporter of pGL3-SV40-hk2-3ARE (hk2-3ARE), human AR expression construct (hAR), Sp1, and c-Jun expression vectors in the indicated combinations. * $P < 0.05$, when compared with the untreated control, or with Mib treatment, or with hk2-3ARE plus Sp1; ** $P < 0.01$, compared with Mib treatment. The parental vectors of pGL3 basic and pGL3-SV40 were included as controls. In each transfection, the pCMV-β-galatosidase (β-gal) was cotransfected to serve as an internal control for transfection efficiency. The cells were incubated with 1 nM of Mib or Mib plus quercetin for a further 24 h after transfection. The normalized, relative luciferase activities (mean ± SE) of at least three independent experiments performed in duplicate were shown

treatment or Mib alone (lanes 4, 5 from left in Fig. 4A). In contrast, when using normal IgG as a negative control, no significant signals were produced in control samples (lanes 7, 8 and 9 in Fig. 4A). Reciprocally, the result showed in Fig. 4B revealed that more AR protein was co-precipitated in c-Jun immunocomplex following quercetin treatment

(lane 6 in Fig. 4B), and a less amount of AR protein was shown in cell lysate treated with Mib alone and no treatment (lanes 4 and 5 in Fig. 4B). In addition, Sp1 protein in lower panel was clearly to be precipitated in c-Jun immunocomplex in cells exposed to quercetin, while less Sp1 was seen in control samples. Finally, the data in Fig. 4C

Fig. 4 In vivo analysis of the associations of c-Jun, Sp1, and the AR Whole cell extracts from cells treated with Mib alone, or Mib combined with quercetin, or remained untreated were prepared. Coimmunoprecipitation (Co-IP) experiments were performed with anti-AR (**A**), anti-c-Jun (**B**), or anti-Sp1 antibody (**C**). Immuno-complexes were fractionated on polyacrylamide gels and the coimmunoprecipitated endogenous c-Jun, Sp1, and AR proteins were detected by using antibodies against c-Jun, Sp1, and AR, respectively. Normal IgG was used as the negative control. Input represents 10% of the whole cell extracts used in the binding experiments
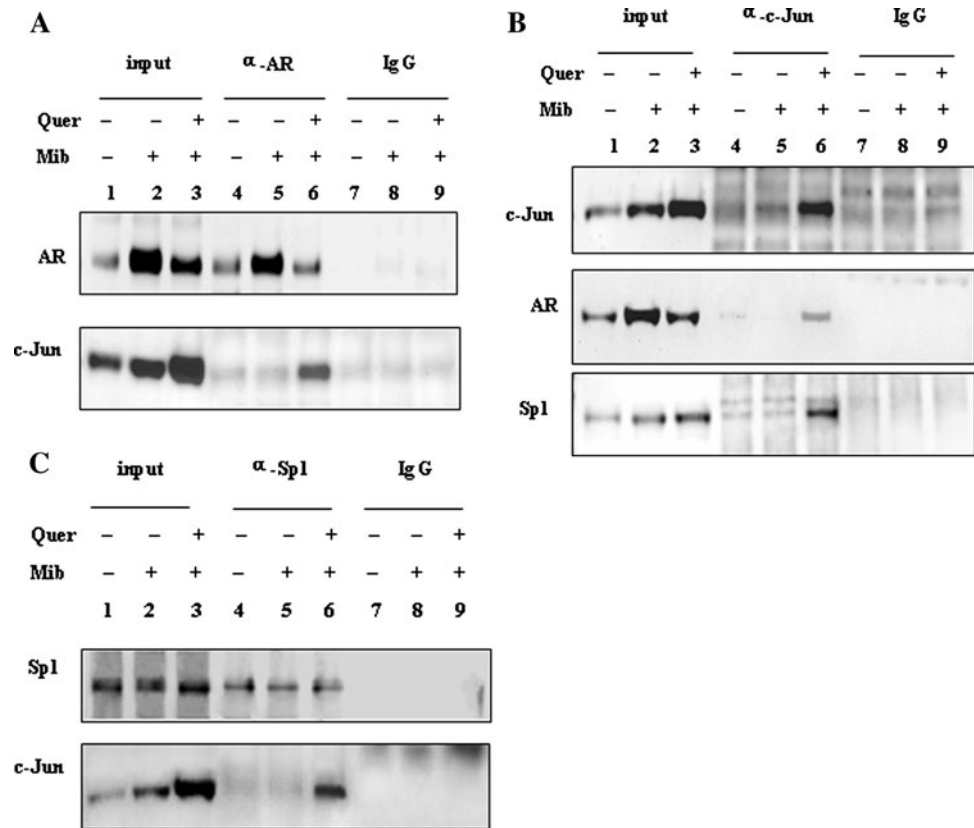
showed that Sp1 was able to pull down more c-Jun protein with it in the quercetin plus Mib treatment than that in the other two groups. Collectively, the results supported the presumption that c-Jun, Sp1 and the AR protein physically interacted in vivo and formed a protein–protein complex in quercetin-treated cells. The associations of these three proteins could result in the repression of AR transcriptional activity on androgen-response genes in prostate cancer cells.

Requirement for induction of c-Jun in attenuation of AR expression and activity

In an attempt to further determine the requirement for induction of c-Jun protein in the quercetin-mediated decrease in AR expression and activity. SP600125, a selectively inhibitor of JNK, was used to reduce c-Jun protein level to test this possibility. The inhibitory effect of SP600125 on JNK activity was verified by a decrease in the phosphorylation level of the JNK substrate c-Jun in cells treated with SP600125. The result in Fig. 5A demonstrated that the phosphorylation level of c-Jun was increased after quercetin treatment in the presence of androgen, while phosphor-c-Jun protein level was decreased in cells pre-treated with SP600125 and quercetin. Next, the effects of the JNK inhibitor on AR expression and function were

examined. The AR protein abundance in response to Mib was blocked by quercetin as shown in Fig. 5B, pretreatment with SP600125 inhibitor partially reversed quercetin-mediated downregulation of AR expression. In addition, androgen-induced production of luciferase activity of PSA promoter was effectively suppressed by quercetin, while inhibitory effect of quercetin on PSA promoter activity was partially restored in cells pretreated with SP600125 as shown in Fig. 5C. These results suggested that induction of c-Jun by quercetin might play an important role in down-regulation of AR expression and transactivity.

Discussion

Like several other plant polyphenols, quercetin shows its inhibitory effect on the expression and function of the AR. Induction of c-Jun by quercetin has been proposed to contribute to the inhibitory mechanism [21, 22]. Moreover, our study indicated that Sp1 stimulated the AR promoter activity and could cooperate with AR to enhance AR transactivation function, whereas quercetin suppresses Sp1-mediated stimulation on the transactivity of AR [23].

In this report, we presented that ectopic expression of Sp1 specifically facilitated AR promoter activity and transactivation, while cotransfection of c-Jun with Sp1
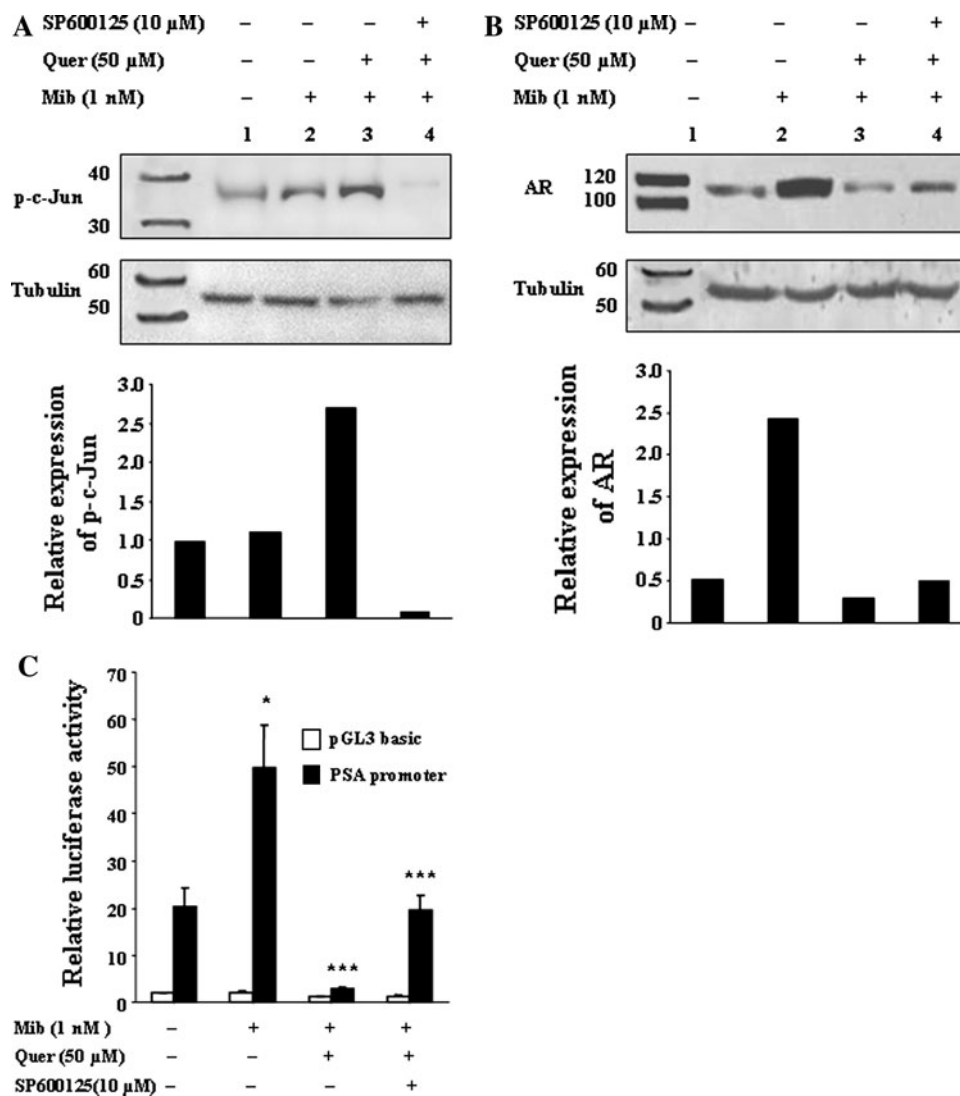
Fig. 5 Induction of c-Jun was required in quercetin-mediated suppression of AR expression and activity. **A** Effect of JNK inhibitor on the change of c-Jun expression in the presence of quercetin. The LNCaP cells were pretreated with 10 μM SP600125 for 1 h, followed by administration of quercetin for 24 h. Protein expression of phosphor-c-Jun was determined by western blot analysis. **B** Western blot analysis of AR expression in cells treated with quercetin or quercetin combined with inhibitor SP600125 as described above. Densitometric measurements were used for analysis of AR and phosphor-c-Jun protein levels which were normalized to β-tubulin. **C** Effect of JNK inhibitor on the AR activity by luciferase reporter assay. LNCaP cells were transfected with pGL3-PSA6 kb promoter (PSA promoter) for 24 h, followed by exposure to quercetin for an additional 24 h after pretreatment with SP600125 for 1 h. The parental vector of pGL3 basic was included as a control, and the pCMV-β-galatosidase was also co-transfected to serve as an internal control for transfection efficiency. The normalized, relative luciferase activities (mean ± SE) of four independent experiments performed in duplicate were shown. * $P < 0.05$, when compared with the untreated control; *** $P < 0.001$, when compared with Mib treatment. Densitometric measurements were used for analysis of phosphor-c-Jun and AR protein levels normalized to β-tubulin

attenuated Sp1-mediated enhancement of the AR promoter activity and AR function. This finding supported the notion that functional interactions of c-Jun, Sp1 and AR were responsible for suppression of the AR transcriptional activity. Previously, we showed that physical interaction of Sp1 and AR was induced in the presence of quercetin [23]. The results of coimmunoprecipitation in this work provided evidence that there were direct associations of c-Jun, Sp1, and AR in quercetin-treatment cells. Therefore,

formation of protein–protein complex resulted in quenching the AR transcriptional function on the regulation of androgen-responsive genes.

The interaction of c-Jun and Sp1 has been reported to be important in the regulation of the p21$^{WAF1/Cip1}$ [28, 29], human 12(S)-lipoxygenase [30], keratin 16 [31], and vimentin [32]. Regulation of sequence-dependent transcription factors may be conveyed by changes in DNA binding activity, protein abundance, association with other

transcription factors, or chemical modification, such as phosphorylation, etc. In case of Sp1, it has been reported to be a positive regulator for AR expression and function [23, 27]. However, neither alterations in Sp1 expression nor detectable changes in DNA binding activity were observed in the presence of quercetin in our study. Functional and physical interaction of Sp1 and AR was further demonstrated by quercetin treatment [23]. In contrast to Sp1, c-Jun, also a coregulator for the AR, was inducible in protein expression by quercetin treatment. The increase in c-Jun expression and its phosphorylated form were significantly sustained for 24 h following quercetin treatment [22]. However, quercetin repressed AR hyperphosphorylation in the presence of androgen as shown before [23]. The alterations in protein abundance and chemical modification of these transcription factors might, at least in part, contribute to the enhancement of their tight binding, subsequently resulted in repression of the AR function, the increased expression level and phosphorylation form of c-Jun might be crucial in this regulation.

In summary, quercetin-mediated inhibition of the AR transcription activity in prostate cancer cells may be caused, at least in part, by the formation of a protein complex containing at least c-Jun, Sp1, and AR. Further investigation will be necessary to examine whether other factors are also involved in this protein complex, and how this protein complex might affect local chromatin structures upon quercetin treatment.

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