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Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells

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Androgens via their cognate receptor may be involved in the development and progression of prostate cancer. The aim of this study was to determine whether tea polyphenols have inhibitory effects on androgen action in an androgen-responsive, prostate cancer cell line, LNCaP. The tea polyphenol, EGCG, inhibited LNCaP cell growth and the expression of androgen regulated PSA and hK2 genes. Moreover, EGCG had a significant inhibitory effect on the androgenic inducibility of the PSA promoter. Immunoblotting detected a decrease in androgen receptor protein with treatments of the tea polyphenols EGCG, GCG and theaflavins. Northern blot analysis showed decreased levels of androgen receptor mRNA by EGCG. Transient transfections demonstrated that EGCG and theaflavins could repress the transcriptional activities of the androgen receptor promoter region. An Sp1 binding site in the androgen receptor gene promoter is an important regulatory component for its expression. This study suggests Sp1 is the target for the tea polyphenols because treatments of EGCG decreased the expression, DNA binding activity and transactivation activity of Sp1 protein. In conclusion, we have described a new property of tea polyphenols that inhibits androgen action by repressing the transcription of the androgen receptor gene. *Oncogene* (2000) **19,** 1924–1932.

Keywords: tea polyphenols; androgen receptor; theaflavins; prostate specific antigen; human glandular kallikrein; Sp1

Introduction

Although androgens are required for growth, differentiation, maintenance and functioning of the prostate (Lindzey *et al*., 1994), they can also be risk factors for prostate cancer development (Brawer and Ellis, 1995; Thompson *et al*., 1995; Pienta and Esper, 1993). Males with 5-α reductase deficiency or castration at early ages do not develop benign or malignant prostate tumors (Brawer *et al*., 1995; Thompson *et al*., 1996; Pienta and Esper, 1993). High levels of plasma androgens may be positively associated with a high incidence of prostate cancer (Dorgan *et al*., 1996; Hamalainen *et al*., 1984; Gann *et al*., 1996). Moreover, the removal of androgens or the blocking of their synthesis can induce apoptosis in both normal and cancerous prostatic epithelia (Lindzey *et al*., 1995; Brawer and Ellis, 1995; Thompson *et al*., 1995). The utilization of androgen deprivation as a treatment for advanced prostate cancer was first demonstrated in 1941 (Huggins and Hodges, 1941) and has become a standard treatment. It is also suggested that the same treatment strategy may be applicable for prostate cancer prevention. To improve the treatment, many new classes of drugs that interfere with androgen production and function have been introduced in recent years (Feigl *et al*., 1995; Kaisary *et al*., 1995; Soloway and Matzkin, 1993; Fleshner and Trachtenberg, 1995). However, in spite of the apparent regression of tumors by hormone therapy, prostate cancer recurs in 1–3 years and becomes hormone refractory with a potentially fatal outcome.

Many molecular mechanisms have been postulated to be responsible for the development of recurrent hormone-refractory tumors. Most of these mechanisms involve alterations in the function of the androgen receptor (AR) and its complex signaling pathways (Koivisto *et al*., 1998). Recent studies have shown that AR is expressed in all stages of prostate cancer, and at least one-third of advanced prostate cancers contain amplified AR genes (Visakorpi *et al*., 1995; Taplin *et al*., 1995; Koivisto *et al*., 1997). Because of mutations in the AR and altered transactivation activities of the receptor in response to non-androgenic steroids, lower concentrations of androgens or even antagonists have been suggested as causative factors for tumor relapse and progression (Culig *et al*., 1998). In addition, the AR could be activated by other signaling pathways without the presence of agonist ligands (Koivisto *et al*., 1998). It is suggested that overexpression or mutation of the AR in prostate cancer cells may promote a growth advantage. Therefore, it has been of great interest to seek more effective means of minimizing or eliminating the function of the AR in order to achieve preventive and/or therapeutic treatments for prostate neoplastic disease.

Liao *et al*. (1995) showed that injections of the green tea polyphenol, (−)-epigallocatechin gallate (EGCG), the most potent component of the green tea polyphenols (GTP), rapidly reduced the size of human prostate and breast tumors in nude mice. These authors suggested that there might be a possible relationship between the higher consumption of green tea and the lower incidence of prostate and breast cancers in some Asian countries. We (Paschka *et al*., 1998) and others (Ahmad *et al*., 1997) have demonstrated that EGCG is capable of inhibiting the cell growth in an androgen-responsive, human prostate adenocarcinoma cell line, LNCaP, at relatively low concentrations. We speculate that the effect of EGCG to inhibit cell growth might be, at least in part, due to diminished androgenic action. In an effort to identify

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naturally occurring inhibitors of the AR, we investigated the possible inhibitory effects of tea polyphenols on androgen action. The tea polyphenols used in this study include; green tea polyphenols (GTP), (−)-epigallocatechin gallate (ECGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-gallocatechin gallate (GCG) and the major polyphenols in black tea, theaflavins. In this report we describe for the first time a novel action of tea polyphenols resulting in a decrease in the expression and therefore function of the AR.

Results

EGCG inhibits LNCaP cell growth and PSA and hK2 expression

To determine if EGCG can inhibit androgen-stimulated growth of LNCaP cells, a dose response assay was performed. LNCaP cells were treated for 4 days with varying concentrations of EGCG with or without a natural androgen, dihydrotestosterone (DHT). An MTS assay was performed to measure cell viability. The upper panel of Figure 1 shows the inhibitory effects of EGCG on LNCaP cells. LNCaP cells treated with DHT alone showed an increase in growth compared to cells without DHT treatment ($P<0.05$). When DHT was present EGCG treatment reduced cell growth significantly even with the lowest concentration of 5 μM. The cells treated with EGCG but without DHT did not show a significant growth inhibition until the highest concentration of 20 μM was used. Cells treated with 20 μM EGCG and DHT showed a similar viability level as control cells without DHT treatment. This indicates that the effects of EGCG were most potent in LNCaP cells treated with androgens, and they inhibited growth to a level similar to cells with no androgen stimulation. The spent media from the dose-response experiments were tested to see if EGCG had an effect on prostate-specific antigen (PSA) secretion levels. PSA is not only the most common biomarker for prostate cancer, but it is also an excellent marker for monitoring androgen action in human prostate cells (Trapman and Cleutjens, 1997; Charlesworth *et al*., 1997; Darson *et al*., 1997; Zhang *et al*., 1997a,b). PSA levels in spent media were determined by immunometric assay and normalized by cell number. As shown in the middle panel of Figure 1, DHT-induced PSA expression is significantly inhibited by EGCG. DHT-induced expression of another prostate specific androgen-regulated protein, human glandular kallikrein (hK2), was also inhibited significantly by EGCG (lower panel, Figure 1).

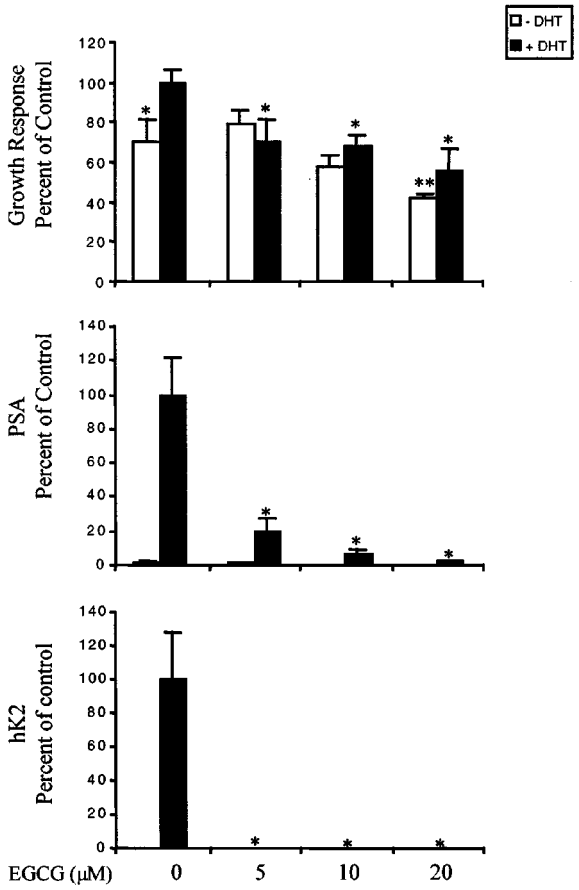

Figure 1 Effects of EGCG on LNCaP cell growth and the expression of PSA and hK2. LNCaP cells were treated with or without indicated concentrations of EGCG and DHT (10 nM) for 4 days. Upper panel: Cell numbers were measured by an MTS assay and expressed as a percentage over the control (DHT alone). Spent media were collected and used for PSA (Hybritech, CA, USA) immunoenzymatic assay or hK2 immunometric sequential (sandwich) assay. Middle panel: PSA and lower panel: hK2 values were normalized to growth response in the upper panel and expressed as a percentage over control (DHT alone). Error bars indicate the standard error of four separate experiments. *$P<0.05$; when compared with the control cells with DHT alone. **$P<0.05$, when compared to the control cells without DHT

EGCG inhibits the expression of the PSA gene at the transcriptional level

It has been well documented that the PSA gene is mainly regulated by androgens at the transcriptional level via interaction of the AR with androgen responsive elements in its promoter (Andrews *et al*., 1992; Montgomery *et al*., 1992). To test whether the inhibitory effect of EGCG on PSA expression occurs at the transcriptional level, a 6 kb PSA promoter-chloramphenicol acetyltransferase (CAT) construct was used for gene transfer experiments in LNCaP cells. In Figure 2 the results show that the CAT reporter gene expression was significantly up-regulated through the PSA promoter with DHT treatment. Treatment with EGCG at 10 and 20 μM significantly decreased the androgenic effect on the PSA promoter. Empty pBLCAT3 vector showed no induction.

Tea polyphenols inhibit AR protein in LNCaP cells

The above results strongly suggest that EGCG can affect the expression of androgen-regulated genes and that these effects may occur at the transcriptional level. Since AR is the most important regulatory transcription factor for the PSA gene, the inhibitory effect of EGCG on the expression of PSA may be occurring via the inhibition of expression and/or function of the AR. Indeed, as shown in Figure 3, EGCG inhibits the expression of the AR protein slightly at 12 h treatment and the inhibition becomes more prominent thereafter. The Ponceau S protein staining was used for a loading and transfer control. A Ponceau S protein band close

to the molecular weight of AR was chosen for illustration purposes. The histogram depicts the relative levels of AR normalized to the Ponceau S band and shown as a percent of the 12 h control lane. The inhibitory effect of EGCG occurred in a time- and dose-dependent fashion. In addition, since EGCG decreased the levels of the AR in the absence of androgen, the inhibitory effect probably occurred independently of AR ligands. Previous studies indicated that phosphorylation states of the AR separated into two or three immunoreactive bands between 110–114 kDa in Western blot analysis depending on the presence or absence of androgens (Blok *et al*., 1996, 1998). Figure 3 shows that all three isoforms of AR were present when cells were treated with DHT, regardless of the presence or absence of EGCG. In contrast, only two isoforms were present when cell cultures were DHT free. This phenomenon suggests that tea polyphenols inhibited AR expression but did not affect its phosphorylation status. Given that EGCG inhibited AR protein levels in the absence of androgens and the evidence that the phosphorylation states were not altered, the data seems to support no involvement of ligand binding activities in the effects of EGCG. Furthermore, a protein stability study was also performed (Kemppainen and Wilson, 1996). The half life of the AR protein was calculated, and the results indicated that the estimated half life of the AR in control cells treated with 10 nM DHT was 12 h which is comparable to the published data (Kemppainen and Wilson, 1996). With EGCG treatment the half life of the AR protein appeared to be very slightly increased to 12.4 h. These studies suggest that the post-translational control of the AR, if any, does not play a crucial role for the tea polyphenol action.

In order to see if EGCG is the only tea polyphenol that can inhibit the AR, another Western blot analysis was performed. When the inhibitory effect of the various tea polyphenols on the expression of AR protein was assessed, EGCG was found to cause the most inhibition of expression, closely followed by GCG (Figure 4). EC and ECG had almost no effet at the concentrations tested. However, the major black tea polyphenols, theaflavins, had a significant inhibitory effect similar to that of EGCG. The inhibitory effects of green tea polyphenols (GTP) and EGCG on the expression of AR protein are shown in the lower panel of Figure 4. In all likelihood, the inhibitory effect of GTP was mainly attributed to EGCG, since EGCG was the major component of GTP accounting for up to 40% of the total mixture.

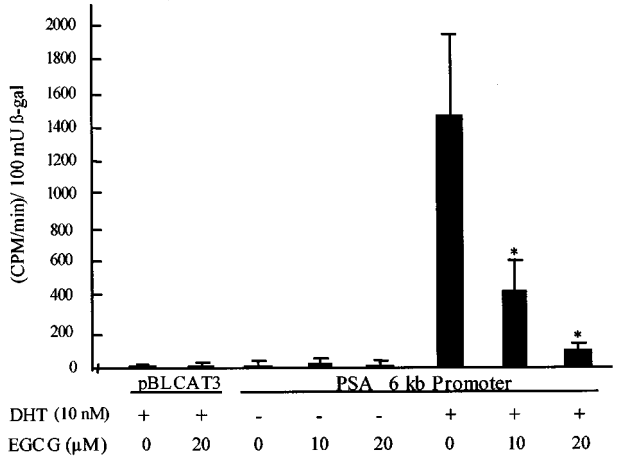

Figure 2 Effect of EGCG on transcriptional activities of the PSA promoter induced by DHT. LNCaP cells were transfected with a 6 kb PSA promoter-CAT reporter plasmid (pBLCAT3-6 kb PSA promoter). A β-galactosidase expression vector was co-transfected to normalize for transfection efficiency. Parental vector (pBLCAT3) was transfected as a control. After transfection, cells were treated with or without indicated concentrations of EGCG and DHT for 24 h. Cell extracts were prepared for CAT and β-galactosidase assays. CAT activity was normalized by β-galactosidase activity and expressed as (c.p.m./min)/100 mU β-gal. Error bars indicate the standard error of three separate experiments

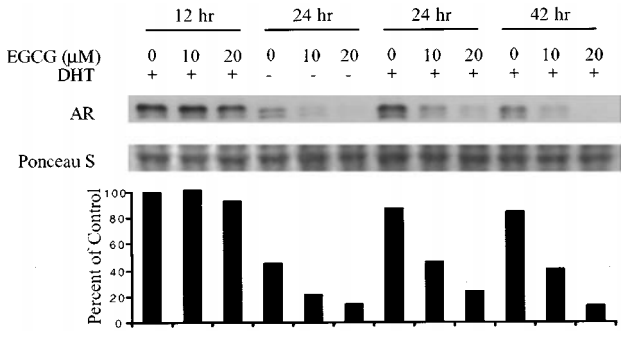

Figure 3 Effects of EGCG on androgen receptor (AR) protein expression in LNCaP cells by Western blotting. Whole cell lysates were prepared from cells treated with or without indicated concentrations of EGCG and DHT (10 nM) at designed times. The upper band shows AR protein levels and the lower band visualizes Ponceau S staining. Ponceau S staining of the blots was performed immediately after electrotransfer, and the band chosen is close to the weight for AR. Note: Due to phosphorylation states, the AR can be detected as doublet or triplet bands at approximately 110 kDa. The histogram represents the levels of AR normalized to the Ponceau S stained protein band and expressed as a percentage of the 12 h no treatment control

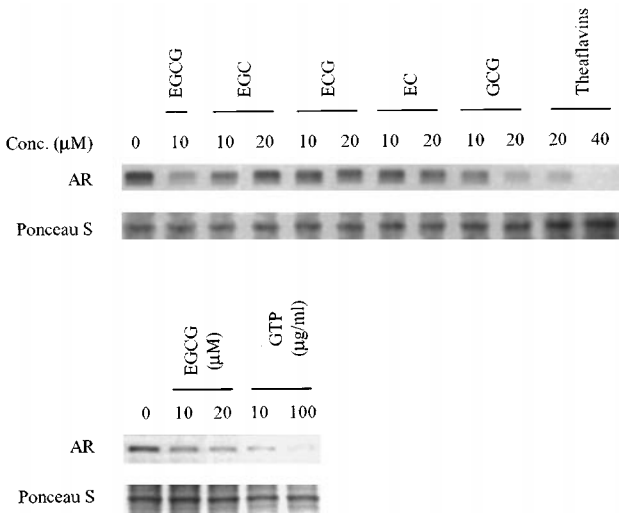

Figure 4 Effects of EGCG and other tea polyphenols on androgen receptor (AR) protein expression in LNCaP cells by Western blot analysis. Upper panel: LNCaP whole cell lysates were prepared 24 h after treatment with different green tea components and black tea theaflavins. Lower panel: LNCaP cells treated with EGCG or green tea polyphenols (GTP) for 24 h were used for preparing whole cell lysates. The Ponceau S band shown as an overall loading control in the figure is a non-specific band close to the molecular weight for AR

EGCG effect on AR mRNA expression

To further elucidate the mechanisms responsible for inhibiting the expression of AR by EGCG, a Northern blot analysis of the steady-state levels of AR mRNA with EGCG treatment was performed. The representative Northern blot shows that EGCG consistently reduced the expression levels of AR mRNA (Figure 5). The average of two separate Northern analyses are depicted in the histogram and shown as a percentage of the control relative to the 12 h time point. Figure 5 shows a reduction in AR mRNA at both concentrations of EGCG and both time points. This result implied that the reduction of AR protein by EGCG was, at least in part, due to the down-regulation of AR mRNA. Note that the AR reduction was about the same at 24 h at both protein and mRNA levels, while at the 12 h point the reduction of AR mRNA by EGCG was higher than that of AR protein, indicating a lagging time between decreases in AR mRNA and protein.

Tea polyphenols inhibit the activity of the 5′-upstream regulatory region of the AR gene

To shed further light on the mechanism of transcriptional down-regulation, we tested whether EGCG had inhibitory effects on the important regulatory regions in the 5′-upstream region of the AR gene. These experiments were carried out using a construct containing 1.4 kb of the AR promoter and the 0.6 kb AR 5′-UTR in transient transfections (AR 2 kb). Figure 6a shows that EGCG and theaflavins effectively reduced luciferase reporter activity in the AR 2 kb construct ($P<0.01$) suggesting that both of these chemicals had an inhibitory effect on the 5′-upstream region of the AR gene. This result correlates well with the observed decrease in AR expression after treatment with these two compounds. As expected EC had no effect on AR 2 kb activity ($P=0.825$).

To further study the transcriptional regulation of AR, the minimal AR promoter core, which exhibited a similar transcriptional activity compared to the long AR promoter (Takane and McPhaul, 1996), was used in transfection experiments. In agreement with the result from the above AR 2 kb studies (Figure 6a), Figure 6b shows the significant inhibitory effects of both EGCG and theaflavins on the AR core promoter region ($P<0.01$) and consistently no effect by EC ($P=0.336$). Together, these studies strongly suggest that the down-regulation of AR expression by EGCG as well as theaflavins was occurring, at least in part, via mechanisms involving transcriptional regulation.

In order to assure the inhibition of the AR promoter activity was specific, a β-galactosidase reporter gene coupled to the CMV immediate early gene promoter/enhancer was transfected into LNCaP cells. Figure 6c shows that neither EGCG nor theaflavins has any effect on the CMV promoter.

Previous studies (Mizokami and Chang, 1994) have demonstrated that the 5′-UTR of the AR gene may

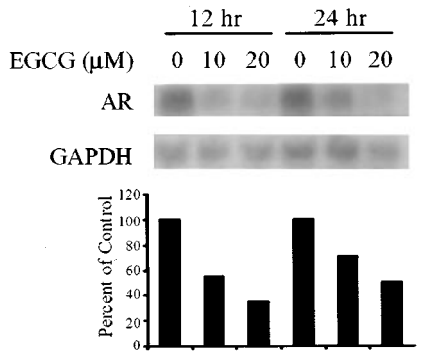

Figure 5 Effects of EGCG on the expression of AR mRNA. LNCaP cells were treated with or without indicated concentrations of EGCG and total RNA was extracted 12 or 24 h after treatment. A representative blot is shown. The upper band shows the AR mRNA levels and the lower band depicts the GAPDH mRNA. The histogram shows the averages of densitometric measurements of two sets of experiments. The AR mRNA values were normalized to GAPDH mRNA values and expressed as percentages of the 12 h control

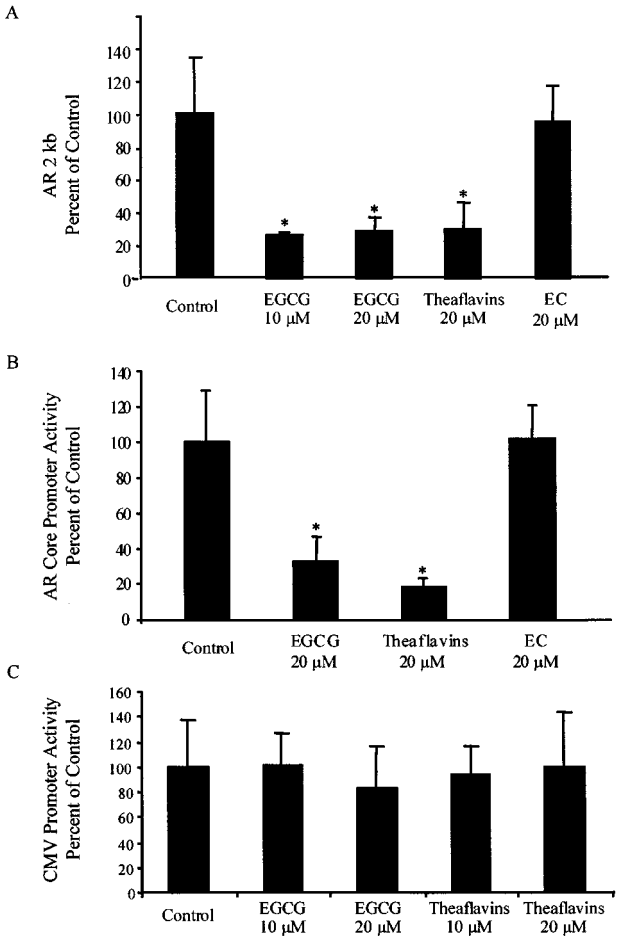

Figure 6 (**a**) Effects of EGCG, EC and theaflavins on the 5′-upstream regulation region of the AR gene (AR 2 kb) by transient transfections in LNCaP cells. After transfection, the cells were treated with EGCG, EC or theaflavins for 24 h. The parental vector (pGL3) was also included as a control vector. Cell extracts were prepared for luciferase and β-galactosidase assays. Luciferase activities were normalized to β-galactosidase. The resulting activities of the AR 2 kb was further normalized to that of pGL3 and presented as a per cent of the control (no EGCG). Error bars show the standard error of three independent experiments. (**b**) Transfections were performed as above except an AR core promoter construct was transfected. (**c**) Transfections were performed using an immediate early gene CMV promoter/enhancer coupled to β-galactosidase. The transfection was performed and the results are reported in the same way as above

positively control the efficiency of its translation. To determine whether the inhibitory effect of EGCG occurs through the AR 5′-UTR, we used an SV40 promoter-driven luciferase reporter vector containing the essential part of the AR 5′-UTR (Mizokami and Chang, 1994) in transient transfection experiments. The vector containing only the SV40 promoter gave a significant decrease in activity when treated with EGCG as did the AR 5′-UTR construct (Figure 7). The lower panel of Figure 7 shows that although the AR 5′-UTR construct also gave a decrease in activity, the extent of the decrease in activity was essentially the same for both the AR 5′-UTR and the parental SV40 promoter vector. This indicates that the SV40 minimal promoter but not the 5′-UTR of the AR gene was responsible for the inhibition of activity seen with EGCG. Taken with the data in Figure 6, this study strongly suggests that the promoter region of the AR gene and not the 5′-UTR was the target for tea polyphenol action.

Effects of EGCG on Sp1

It has been shown that the Sp1 transcription factor and its DNA binding motif play a role in regulation of transcriptional activities for the AR promoter as well as the SV40 minimal promoter (Ryu *et al*., 1999). The decrease in activity in the SV40 promoter could indicate that EGCG had an effect on Sp1. A gel bandshift assay was performed to see if EGCG can affect the Sp1 binding ability for the AR promoter's Sp1 motif. LNCaP nuclear extract was incubated with a probe for the Sp1 motif with or without antibodies and competitors (Figure 8). The bandshift experiment showed that Sp1 binding could be competed out by unlabeled Sp1 DNA and supershifted by an Sp1 specific antibody. EGCG was able to significantly decrease the Sp1 DNA binding activity. Non-specific antibody could not cause supershifting and unrelated DNA competitor did not have specific competitive effects (Figure 8).

Since Sp1 DNA binding was decreased by EGCG, a Western blot analysis was performed to see if Sp1 protein levels are decreased by EGCG. Figure 9a shows that EGCG was able to decrease Sp1 protein in nuclear and whole cell extracts. To further demonstrate that a decrease in Sp1 protein mediates the inhibitory effects of EGCG on transcriptional activity of the AR promoter, a transfection experiment was performed. Constructs containing three copies of the AR Sp1 motif and three copies of a mutated AR Sp1 motif were transfected into LNCaP cells for 24 h and then treated for 24 h with EGCG. Note that the mutation of the AR Sp1 motif abolished its transcriptional activity, which was not further affected by EGCG (Figure 9b). In contrast, EGCG could significantly decrease the transcriptional activity of the wild type AR Sp1 motif (Figure 9b). This data taken together indicates that EGCG decreased the AR's functions by decreasing its expression possibly through decreasing the level of the transcription factor Sp1.

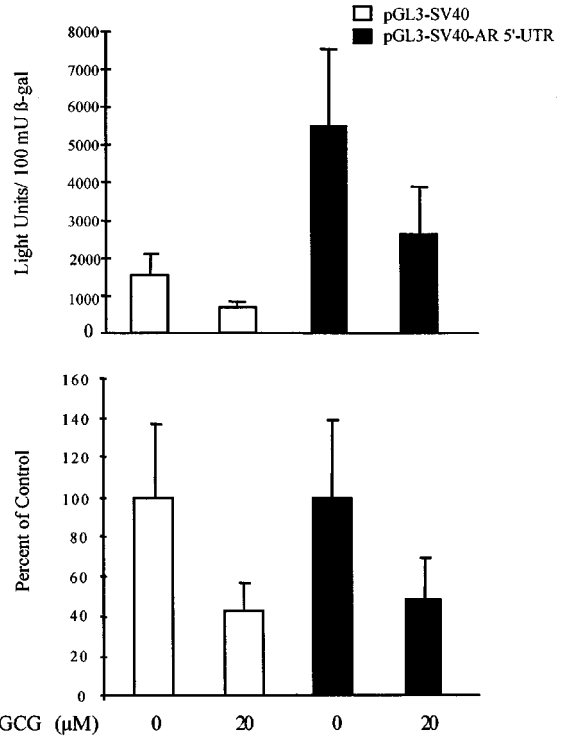

Figure 7 Effects of EGCG on the 5′-UTR of AR gene by transient transfections. Vectors containing SV40 promoter-AR 5′-UTR-luciferase reporter gene and CMV-β-galactosidase were used to transfect LNCaP cells and treated with or without EGCG for 24 h. The parental vector (pGL3-SV40) was also used as a control for co-transfection. Cell extracts were prepared for luciferase and β-galactosidase assays. Luciferase activities were normalized to β-galactosidase. Six independent experiments were presented. The upper panel shows the β-galactosidase normalized value of luciferase activity; the lower panel shows the data in the upper panel as a per cent of the control (no EGCG treatment) of each construct

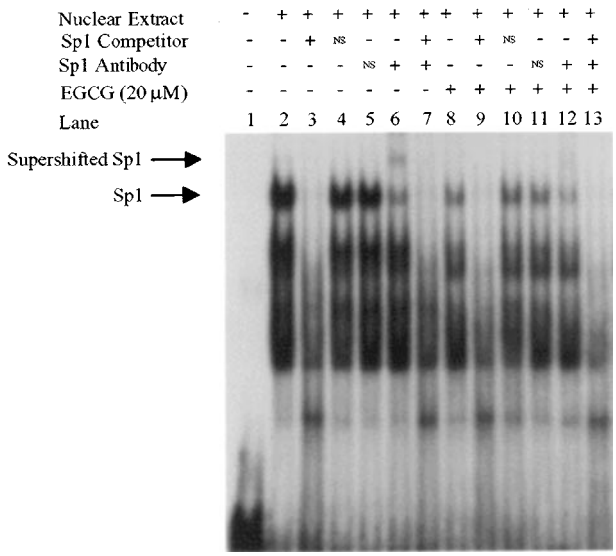

Figure 8 Gel shift assay to show EGCG effects on the binding of the AR Sp1 motif. ^{32}P-labeled AR Sp1 motif oligo was incubated with LNCaP nuclear extract treated with or without EGCG. Lane 1 is free probe. Lanes 2 and 8 are the probe with nuclear extract alone. Lanes 3 and 9 have Sp1 cold competitor in 100-fold excess. Lanes 4 and 10 contain a non-specific AR E box motif competitor. In lanes 5 and 11 the binding reactions were incubated with non-specific antibody. In lanes 6 and 12 the binding reactions were incubated with an Sp1 specific antibody. Lanes 7 and 13 show 100-fold excess of Sp1 cold competitor and Sp1 antibody. Lanes 8–13 are treated with 20 μM EGCG. The arrows indicate the Sp1 specific band and the supershift with a specific antibody, respectively. NS: non-specific

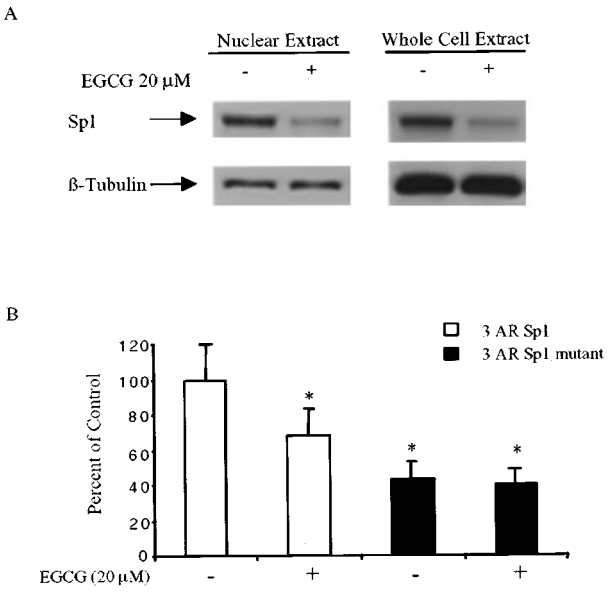

Figure 9 Effects of EGCG on Sp1 protein levels and its transactivation activity. (**a**) A Western blot analysis of Sp1 protein in nuclear and whole cell extracts of cells treated with or without EGCG. The upper band depicts Sp1 protein levels and the lower band gives β-tubulin as a loading control. (**b**) A transfection in LNCaP cells in the presence or absence of EGCG treatment with a pGL3 SV40 promoter plasmid or the plasmid containing three copies of the mutant or wild type AR Sp1 motif. Three separate experiments were performed. The results were normalized to co-transfected β-galactosidase activities. The final activity was presented as a per cent of the level of the wild type Sp1 construct with no EGCG treatment

Discussion

There have been a number of *in vitro* and *in vivo* studies demonstrating that certain tea components have potential chemopreventive effects on prostate cancer through various mechanisms including inhibition of cell growth (Blot *et al*., 1996; Liao *et al*., 1995; Paschka *et al*., 1998), induction of apoptosis (Paschka *et al*., 1998; Ahmad *et al*., 1997), cell cycle arrest (Ahmad *et al*., 1997), inhibition of type 2 5-α-reductase (Liao and Hiipakka, 1995) and *in vitro* and *in vivo* inhibition of testosterone-mediated induction of ornithine decarboxylase (ODC) (Gupta *et al*., 1999). More understanding of the biological properties of tea polyphenols is needed before the clinical application of tea polyphenols as an intervention agent for prostate cancer is considered. Also, it is worthwhile to mention that although the concentrations of tea polyphenols in plasma have been reported to be relatively low after oral administration, these substances seem to still be able to inhibit *in vivo* androgen stimulated expression of ODC gene (Gupta *et al*., 1999). It is not clear at the present time how the low concentrations of tea polyphenols in plasma can effectively affect androgen effects on ODC. However, our study for the first time has identified a novel molecular mechanism by which some tea polyphenols can cause an inhibition of androgen action. Certain tea polyphenols can repress AR expression at the transcriptional level possibly by decreasing Sp1 protein levels.

The early development and growth of prostate cancer is highly dependent on androgens. However, androgens themselves are not necessarily involved in the activation of AR function in endocrine therapy-resistant prostate cancers. In recurrent prostate cancer, the mutated but functionally intact AR can be bound by lower concentrations of androgens or other ligands as well as overexpressed AR. This might be a crucial factor in stimulating androgen-independent cancer cell growth (Koivisto *et al*., 1996; 1998; Culig *et al*., 1998; Visakorpi *et al*., 1995; Gregory *et al*., 1998; Labrie *et al*., 1993). Moreover, a recent study by Gregory *et al*. (1998) has demonstrated that the human prostate cancer xenograft, CWR22, grown in testosterone-stimulated nude mice regresses after castration but recurs after 5–6 months in the absence of testicular androgen. Like human prostate cancer that recurs during androgen deprivation therapy, the recurrent CWR22 expresses high levels of AR as well as AR-regulated genes even in the absence of testicular androgen. The authors also point out that a network of AR-regulated genes is likely to play a role in driving the growth of androgen-independent prostate cancer potentially via alternative AR activation pathways (Gregory *et al*., 1998). Therefore, only aiming at reducing the circulating levels of androgens or blocking agonist binding to AR without actually down-regulating AR expression could be one possible reason for the overall failure of endocrine therapy. Thus, the novel property of tea polyphenols to inhibit AR expression thereby limiting its availability, apart from their other known or unknown anti-cancer properties, could have a great potential for preventing or even treating relapsed prostate cancers.

It is well recognized that androgens can enhance AR protein levels by increasing the half-life, as well as by stimulating the phosphorylation (Zhang *et al*., 1999; Zhou *et al*., 1995; Kemppainen *et al*., 1992; Krongrad *et al*., 1991) of the AR. It has been shown that agonist-bound AR is more resistant to *in vitro* proteolytic digestion than an unbound AR, indicating a conformational change of the AR induced by androgens. Thus, a conformational change may expose sites for phosphorylation by protein kinases (Blok *et al*., 1996). It has been suggested that phosphorylation may play a role in many diverse processes for nuclear receptors including ligand binding, nuclear translocation, dimerization, DNA binding, and protein-protein interactions (Blok *et al*., 1996). Androgen-induced phosphorylation of the AR results in an altered migration pattern on a SDS–PAGE gel, as reported previously (Van Laar *et al*., 1991) and suggested in this study. It has also been suggested that EGCG may inhibit certain protein kinases (Stoner and Mukhtar, 1995). However, our study does not seem to suggest that phosphorylation of the AR is affected by tea polyphenols (Figure 3). Also, this study suggests that the degradation rate of AR protein is not enhanced by tea polyphenols. Furthermore, the reduction of AR protein levels by EGCG appears to be independent of androgens (Figure 3).

To answer the question: what is the molecular mechanism by which tea polyphenols modulate the expression of the AR, we studied the effect of EGCG on AR mRNA steady state levels. Northern analysis showed a significant reduction in AR mRNA by EGCG treatment indicating a potential transcriptional regulation of the AR gene. Our transient transfection data showed more definitively that GTP, EGCG and theaflavins were capable of inhibiting AR promoter activity. This study also indicates that the AR 5′-UTR is not the inhibitory site for tea polyphenols. Although other unknown mechanisms may exist for the action of

tea on androgen receptor expression, the data from our present study provides considerable evidence to support a mechanism of transcriptional down-regulation.

It has been demonstrated (Takane and McPhaul, 1996; Chen *et al*., 1997) that an Sp1 binding site within the AR core promoter region with its binding factors may play an important role for the basal activity of the AR promoter. Sp1 is the prototype of Sp/XKLF family proteins (e.g., Sp2, Sp3, Sp4, BTEB, BTEB2, TIEGs and the Kruppel-like factors, etc.) and is a zinc finger transcription factor that binds to a GC-rich DNA motif in a wide range of genes (Philipsen and Suske, 1999). Although it was believed that Sp1 only mediated those constitutively activated genes, recent evidence shows that Sp1 in cooperating with many other factors can mediate inducible regulation of many genes (Philipsen and Suske, 1999). Even though Sp1 is involved in the expression of genes related to cell proliferation and other important cellular processes (Philipsen and Suske, 1999; Alroy *et al*., 1999), mammalian cells lacking Sp1 protein can grow and differentiate *in vitro*. This may be because perhaps some of the functions of Sp1 can be replaced by other Sp proteins. On the other hand, since Sp1 can regulate the expression of many critical genes, the decrease in this protein by tea polyphenols could somewhat decrease the growth rates of prostate cells. This may explain why the growth of LNCaP cells in the absence of androgens can be further inhibited by high concentrations of EGCG. Alternatively, a yet unidentified factor other than the AR critical for cell growth could be affected by high concentrations of EGCG. Nevertheless, this study has identified two transcription factors, AR and Sp1 that can be affected by some tea polyphenols, implying that their downstream target genes and subsequently the functions of these gene products can be affected by tea polyphenols.

Materials and methods

Cell cultures and treatments

The human prostate cancer cell line LNCaP was obtained from The American Type Culture Collection (Rockville, MD, USA) and propagated in 24-well, 60 or 100 mm culture dishes at the desired density in RPMI 1640 (Celox, St Paul, MN, USA) medium supplemented with 5% fetal bovine serum (FBS) (Biofluids, Rockville, MD, USA) at 37°C and 5% CO_2 until the confluency reached 50–75%. The cells were treated with tea polyphenols at designated concentrations with or without dihydrotestosterone (DHT) at physiological concentrations (10 nM) in serum- and phenol red-free RPMI 1640 medium. Tea polyphenols used in the study include green tea polyphenols (GTP) (LTK, St Paul, MN, USA), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-gallocatechin gallate (GCG) and black tea theaflavins (Sigma, St Louis, MO, USA). Tea polyphenols were dissolved in phosphate buffered saline. DHT was dissolved in 100% ethanol as a stock solution. Equivalent amounts of vehicle solvents were used as controls.

Cell growth and PSA and hK2 protein expression

LNCaP cells were seeded at 2×10^4 cells/well in 24 well plates. After 48 h the medium was changed to serum-free RPMI 1640 medium, and the cells were incubated for an additional 24 h to deplete endogenous steroid hormones prior to experiments. Cells were then treated with different concentrations of EGCG either with or without 10 nM DHT. After a 4 day incubation, spent media were harvested, and levels of PSA and hK2 protein in spent media were quantified by an immunometric assay as described previously (Hsieh *et al*., 1997). Cell density was quantified by MTS assay as per manufacturer's instructions (Promega, Madison, WI,, USA). The protein levels of PSA and hK2 were normalized by cell density measurements and expressed as a percentage over control (DHT alone).

Plasmid construction

To generate plasmid constructs containing the AR core promoter (AR−74/+87) (Takane and McPhaul, 1996) or the AR 5′-UTR fragment (AR+21/+377) (Mizokami and Chang, 1994), the pGL3-luciferase plasmid containing 1.4 kb promoter+0.6 kb 5′-UTR (AR−1380/+577) of the human AR gene (kindly provided by Dr Donald Tindall, Mayo Foundation, Rochester, MN, USA) was used as a template for polymerase chain reaction (PCR). The PCR products were digested and gel-purified. The AR core promoter (AR−74/+87) fragment was ligated into a pGL3-luciferase vector between *Xho*I and *Hind*III cutting sites. The AR 5′-UTR fragment (AR+21/+377) was ligated into a pGL3-SV40 minimal promoter-luciferase vector precut with *Hind*III restriction enzyme and the fragment was inserted between the SV40 minimal promoter and the luciferase reporter gene. A 6 kb PSA promoter (Spitzweg *et al*., 1999)-chloramphenicol acetyltransferase (CAT) construct and the parental empty vector pBLCAT3 were also used for transient transfection assays. Double stranded oligonucleotides containing three copies of wild type or mutant AR Sp1 motif (the upper strand sequence of wild type AR Sp1 oligo: 5′-TCG AGC GAG TCG GGT CCC GCC CCC ACC GGG CCG GCG AGT CGG GTC CCG CCC CCA CCG GGC CGG CGA GTC GGG TCC CGC CCC CAC CGG GCC GGA-3′; the upper strand sequence of mutant AR Sp1 oligo: 5′-CGC GTC CGG CCC GGT GGA AAA AAG ACC CGA CTC GCC GGC CCG GTG GAA AAA AGA CCC GAC TCG CCG GCC CGG TGG AAA AAA GAC CCG ACT CGC-3′) were ligated into the pGL3-SV40 promoter vector at the *Mlu*I and *Xho*I sites. All the above plasmid constructs made were confirmed by DNA sequencing and used for transient transfection assays.

Transient transfection assay

LNCaP cells were seeded at 3.75×10^5 cells in 60 mm dishes and grown under the conditions described above. Cells were transfected with 4 μg pBLCAT3 containing a 6 kb PSA promoter-CAT reporter using a liposome containing dimethyldioctadecyl-ammonium bromide and L-α-lecithin (4 : 10 w/w) as reported previously (Zhang *et al*., 1997a) under serum-free conditions, β-galactosidase (β-gal) CMV vector (0.2–0.35 μg) was co-transfected to normalize transfection efficiency. pGL3 containing a 1.4 kb promoter+0.6 kb 5′-untranslated region (5′-UTR)-luciferase reporter gene construct, pGL3-0.161 kb AR core promoter-luciferase construct, pGL3-SV40 promoter-0.35 kb AR 5′-UTR-luciferase construct or pGL3-SV40 promoter 3 AR Sp1 mutant and wild type (2.5–4 μg) were used for transient transfections. The parental vectors pBLCAT3, pGL3, and pGL3-SV40 promoter were used as controls. Twenty to 24 hours after transfections, cells were treated with tea polyphenols, DHT or vehicles for an additional 24 h in serum- and phenol red-free RPMI 1640 medium. Cell extracts were prepared from duplicate plates and used for CAT and β-gal assays according to published methods (Zhang *et al*., 1997a,b) or for luciferase assays (Promega, Madison, WI, USA). At least three independent transfections were performed.

Western blot analysis

For immunoblotting of the AR, LNCaP cells were grown under the same conditions described above and treated with or without tea polyphenols in the presence or absence of DHT. Whole cell lysates were prepared using RIPA lysis buffer containing 1 × PBS, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS. Nuclear extracts for gel bandshift assays below were also used in this analysis. Freshly prepared protease inhibitors (100 μg/ml PMSF, 30 μl/ml aprotinin, 1 mM sodium orthovanadate) were also included. Protein content was quantified by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Eight per cent polyacrylamide reducing SDS gels were run and electrotransferred onto nitrocellulose membrane (Bio-Rad). The nitrocellulose membranes were immediately stained by a working solution of Ponceau S (0.1% Ponceau S, 5% acetic acid) to show loading and transfer efficiency of proteins and were photographed. The blots were then blocked overnight at 4°C with 5% non-fat milk in TBST buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20, pH 8.0) prior to incubation with a monoclonal antibody against AR (1 : 2000 dilution; PharMingen, San Diego, CA, USA) or Sp1 (1 : 2000 dilution, Santa Cruz, Santa Cruz, CA, USA) for 1 h at room temperature. Membranes were incubated with an anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (1 : 3000 dilution; Amersham, Arlington Heights, IL, USA) at room temperature for 1 h. Blots were washed between each step with TBST and visualized by enhanced chemiluminescence substrate (ECL, Amersham Corporation, Arlington Heights, IL, USA) then exposure to X-ray film.

Northern blot analysis

LNCaP cells (3×10^5 cells/ml) were grown under the same conditions described above and treated with or without EGCG. Cells were harvested at indicated times, and total RNA was extracted by the guanidine thiocyanate method (Chomazynski and Sacchi, 1987). Equal amounts of RNA (35 μg/lane) were fractionated in the presence of ethidium bromide by denaturing agarose gel electrophoresis in 1 × borate buffer and transferred to a Zeta Probe membrane (Bio-Rad). Membranes were pre-hybridized for 4 h at 65°C using hybridization buffer (7% SDS, 1 mM EDTA, 0.25 M $Na_2H_2PO_4$ and 0.25 M Na_2HPO_4) and then hybridized overnight at 65°C with a 0.5 kb ^{32}P-labeled AR probe which was generated by digesting the full length hAR cDNA with *Bam*HI followed by gel-purification. Unbound probe was washed from membranes in successive washes with 1 × SSC plus 0.05% SDS and 0.1 × SSC plus 0.1% SDS. Fully washed membranes were exposed to X-ray film for 12–24 h at −70°C. mRNA levels of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also measured on the same membrane for normalization purposes. The exposed films were scanned by a densitometer. The density values of AR mRNA were normalized by the values of GAPDH at different concentration of EGCG at 12 or 24 h.

Gel band-shift assay

The following oligonucleotides were synthesized and annealed to their complementary oligonucleotides (not shown) to form a double stranded DNA: AR Sp-1 (upper strand: 5′-TCG GGT CCC GCC CCC ACC GGG C-3′) and AR E-box (upper strand: 5′-GGA GAG CAA ATG CAA CAG-3′) as a non-specific probe. These sequences are located in the core region of the AR gene (Takane and McPhaul, 1996). The band shift assay was described previously (Zhang *et al*., 1997a,b). Briefly, the above double stranded AR Sp1 oligonucleotides were radiolabeled using α-^{32}P-dCTP with Klenow enzyme to a specific activity of 8×10^7 to 8×10^8 c.p.m./μg. *In vitro* DNA binding was performed by incubating the above nuclear extract (5 μg) in a buffer containing 20 mM HEPES, pH 7.9, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 12% (w/v) glycerol, 4 mM DTT, 1 μg poly (dI:dC) with or without 100-fold molar excess of an unlabeled Sp1 probe or AR E-box DNA for 10 min at room temperature prior to receiving 20–30 fmol of a labeled ds-oligonucleotide probe for an additional 10 min of incubation. Sp1 specific antibody (0.4 μg/lane) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) was included in some of the reactions with an additional 30 min incubation on ice. Finally, the above reaction mixtures were electrophoresed in a prerun 5% polyacrylamide (acrylamide:bisacrylamide, 29 : 1 w/w) gel with 0.5 × Tris-borate EDTA buffer. Gels were dried and autoradiographed.

Immunoprecipitation

LNCaP cells were seeded in 100 mm plates (9×10^5 cells/plate) and incubated under the same conditions described above for 72 h until cell confluency reached 70–80%. The media was washed and changed with 4 ml methionine-free RPMI 1640 (Gibco, Fredrick, MD, USA) containing 10 nM DHT and 2% dialyzed fetal calf serum. The cells were incubated at 37°C, 5% CO_2 for 30 min. The spent media was aspirated and fresh media was added containing 100 μCi/ml [^{35}S]Methionine. The cells were incubated for another 30 min. Cells were then treated with or without 20 μM EGCG in phenol red-free RPMI 1640 in the presence of 10 nM DHT. Cells were harvested and lysed as per the Western blotting methods at 8, 12, 14, 16 and 18 h. Protein contents of cell lysates were quantified using Bio-Rad DC protein assay. The lysates were stored at −80°C until use. The lysates (100 μg protein/sample) were then precleared by incubating with 2 μg Mouse IgG together with 40 μl protein G PLUS/Protein A-Agarose and incubated 2 h at 4°C with rotation. The samples were centrifuged at 1500 r.p.m. to pellet the agarose. The collected supernatants were incubated with 3 μg androgen receptor antibody and 60 μl protein G PLUS/Protein A-Agarose per sample at 4°C with rotation overnight. Then the samples were centrifuged at 2500 r.p.m. for 15 min at 4°C. The pellets were washed in chilled lysis buffer (1% Triton X-100, 5 mM EDTA, 5 mM Na azide, 10 mM NaF, 10 mM phosphate, 10 mM Na pyrophosphate, 150 mM NaCl, 10 mM Tris, pH 7.5 and 1 × Protease Inhibitor Cocktail) three times, in RIPA buffer two times, and in lysis buffer for the last wash. Washed pellets were resuspended in 50 μl of 1 × SDS–PAGE sample buffer (2% SDS, 0.03% Bromophenol Blue, 0.3% (v/v) β -Mercaptoethanol, 10% glycerol and 67 mM Tris, pH 6.8). The bound immunocomplex was eluted by boiling the pellets for 2 min and analysed by 8% SDS–PAGE. Gels were dried and autoradiographed. The AR bands were quantitated by digital camera and the points plotted. A slope was calculated by a line equation in Microsoft Excel and the half life of the AR was determined.

Statistics

Statistical analysis was performed using the Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

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