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2,3,7,8-Tetrachlorodibenzo-p-dioxin Blocks Androgen-Dependent Cell Proliferation of LNCaP Cells through Modulation of pRB Phosphorylation

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ABSTRACT

Cell-cycle regulatory events associated with inhibition of androgen-dependent cell proliferation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were studied in the human-derived LNCaP cell line. TCDD blocked the G_1 to S transition of LNCaP cells synchronized in G_0/G_1 when these cells were induced to reinitiate cell-cycle progression by dihydrotestosterone (DHT). Western blot analyses of these cells revealed altered expression levels of G_1 regulatory proteins, including increases in hypophosphorylated retinoblastoma protein and concomitant decreases in cyclin D1. p21$^{WAF1/CIP1}$, which is involved in the assembly of cyclin D1/cyclin-dependent kinase-4 complexes, was increased by DHT or TCDD when each compound was administered singly but was reduced to background levels in cells simultaneously treated with DHT and TCDD. Reporter gene assays revealed the presence of several Ah receptor response-element motifs in the promoter and first intron of the p21$^{WAF1/CIP1}$ gene that respond to TCDD-mediated Ah receptor activation independently of p53. Transcription studies showed that activation of aryl hydrocarbon receptor blocks androgen-dependent gene induction in LNCaP cells as well as in African green monkey CV-1 cells. These data point to at least two mechanisms whereby TCDD blocks androgen receptor function: 1) by blocking androgen-induced cell proliferation through modulation of the expression and activities of regulatory proteins controlling cell-cycle progression; and 2) by squelching androgen receptor-mediated gene transcription through receptor cross-talk, possibly involving competition for coregulators or by direct protein interaction.

Depending on cell type and lineage, exposure to TCDD often leads to multiple diverse responses, including changes in cell proliferation and differentiation. Most, if not all, of the effects of TCDD are mediated through binding to a ligand-activated transcription factor known as the Ah receptor. In brief, TCDD binds to the AHR and facilitates the release of bound 90-kDa heat-shock proteins, resulting in receptor activation. Subsequent to this release, liganded AHR translocates to the nucleus, in which it heterodimerizes with ARNT. AHR and ARNT are similar in structure and belong to the basic helix-loop-helix/Per-Arnt-Sim class of transcription factors. AHR/ARNT heterodimers bind to specific AhRE regulatory sequences in the promoter regions of AHR-responsive genes. Once bound, the heterodimer functions as a transcription factor to enhance the expression of AHR-responsive genes, such as the cytochrome P450 genes CYP1A1, CYP1B1, and CYP1A2 and several other genes encoding phase II detoxification enzymes, such as NAD(P)H-dependent quinone oxidoreductase-1 and others (Hankinson, 1995). It is becoming increasingly evident, however, that the AHR has functions outside of xenobiotic metabolism and transcriptional up-regulation that include roles in cellular growth, development, and differentiation (Puga et al., 2002).

Androgens, such as testosterone, are normally secreted by the Leydig cells of the testes and regulate growth and development in the male reproductive system. During development, androgens are required for proper morphogenesis of

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AHR, aryl hydrocarbon receptor; AhRE, Ah receptor response element; AR, androgen receptor; ARE, androgen receptor response element; ARNT, aryl hydrocarbon receptor nuclear translocator; CDT, charcoal-dextran–treated fetal bovine serum; DHT, dihydrotestosterone; ER, estrogen receptor; FBS, fetal bovine serum; IMEM, improved minimal essential medium; PSA, prostate-specific antigen; RB, retinoblastoma; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; C_T, cycle threshold; PAGE, polyacrylamide gel electrophoresis; R1881, methyltrienolone; BrdU, 5-bromo-2′-deoxyuridine; HSV, herpes simplex virus; cdk, cyclin-dependent kinase.

male accessory sex organs, imprinting responses of the accessory sex organs for later androgenic stimulation, initiating spermatogenesis, and directing the male pattern of sexual differentiation of the central nervous system. Androgens function through the androgen receptor to regulate gene expression in the testes, seminal vesicle, vas deferens, and the prostate. The mechanism by which androgens function through the AR follows a pattern similar to that described for TCDD and the AHR (Lee and Chang, 2003). Like AHR, members of the steroid hormone-receptor family function as ligand-activated transcription factors. Upon binding of DHT, the ultimate agonist in prostate, the AR undergoes a conformational change that facilitates the release of the 90-kDa heat-shock protein and the unmasking of DNA binding domains, which promote the association of androgen-AR complexes with AREs located in the promoter regions of androgen-responsive genes such as human prostate-specific antigen (PSA). AR binding leads to transcriptional activation of some genes and repression of others. Therefore, any disruption of normal androgen function can lead to adverse and deleterious effects on male reproduction (Griffiths et al., 1997).

In male rats exposed in utero or lactationally (Moore et al., 1985; Mably et al., 1992; Theobald and Peterson, 1997), TCDD decreases growth of the testis, epididymis, and accessory sex organs (seminal vesicles and prostate), alters testicular and epididymal morphology, decreases daily and ejaculated sperm numbers, and impairs reproductive performance. These TCDD effects have been attributed to decreases in circulating androgen levels caused by alterations in enzymes that catalyze testosterone synthesis or its conversion to DHT in androgen target tissues or by a reduction in Leydig cell number, size, and function (Johnson et al., 1992). However, other studies have shown that TCDD exposure does not correlate with decreases in circulating androgen or in androgen receptor levels (Roman et al., 1995, 1998), which suggests that TCDD does not alter the synthesis or metabolism of androgen to cause antiandrogenicity. It is more likely that TCDD acts directly on target organs to disrupt androgen-induced responses at the level of gene transcription, cell proliferation, or both.

Epidemiological studies in humans and in in vivo studies in experimental animals suggest that there is cross-talk between AHR and steroid hormone receptor signal transduction pathways, including the estrogen, androgen, and thyroid hormone receptors (Porterfield, 1994; Wang et al., 1998; Jana et al., 1999). In female mice and rats, TCDD inhibits estrogen-induced uterine growth, peroxidase activity, c-Fos protoncogene mRNA levels, and the expressions of the estrogen, progesterone, and epidermal growth factor receptors (Astroff and Safe, 1990). The mechanism by which TCDD inhibits ER-mediated gene transcription has been established for at least two genes: cathepsin D (Kharat and Saatcioglu, 1996; Wang et al., 2001) and pS2 (Zacharewski et al., 1994). It has been shown that the TCDD-activated AHR binds to inhibitory dioxin-response elements overlapping estrogen response elements in the promoter regions of these two genes. By this mechanism, AHR acts as an antiestrogen blocking the ability of the ER to bind DNA and activate gene transcription. AHR has also been shown to interact directly with ER (Klinge et al., 2000) and to modulate ER-mediated estrogenic signaling (Ohtake et al., 2003).

TCDD was found to block normal and androgen-dependent cell proliferation in human derived androgen-dependent LNCaP cells (Jana et al., 1999). In addition, reciprocal transcriptional interference was observed between androgen- and TCDD-mediated pathways, although the mechanisms responsible for such interference were not defined. The present study extends that work and identifies molecular events critically associated with TCDD-mediated disruption of androgen receptor function in LNCaP cells.

Materials and Methods

Cell Culture and Treatments. LNCaP, Hepa-1, CV-1, and MCF-7 cell lines were purchased from American Type Culture Collection (Manassas, VA) and maintained in a 5% CO_2 atmosphere. For normal growth, LNCaP cells were maintained in phenol red-free improved minimum essential medium (IMEM) (Biosource International, Camarillo, CA) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). Hepa-1, MCF-7, and CV-1 cells were maintained in α-minimal essential medium supplemented with 5% FBS (Invitrogen) and 100 U/ml penicillin/streptomycin. For experiments, LNCaP cells were seeded in IMEM supplemented with 5% CDT (Hyclone) depleted of steroids but containing growth factors.

Plasmid Constructs. The PSA61luc reporter construct (Cleutjens et al., 1997) contains 6.1 kb of the human *PSA* promoter. To generate PSA-AREluc and PSA-ARE-AhREluc, the full-length PSA61luc construct was digested with HindIII and BglII or KpnI and the respective 556-bp and 1.5-kb fragments thus formed were subcloned into the pGL3Basic promoterless luciferase reporter vector (BD Biosciences Clontech, Palo Alto, CA). PSA-AhRE-AREluc was constructed by digesting the full-length PSA61luc reporter construct with HindIII and KpnI and subcloning the 1.5-kb fragment into the PGL3Basic reporter (BD Biosciences Clontech). ARR2PB-LUC is a reporter containing the androgen-responsive region of the rat probasin gene promoter; pSG5-AR is a eukaryotic expression of the wild-type human androgen receptor. A 399-bp fragment of the first intron of the human p21 gene, containing seven AHRE sites and a 1.3-kb fragment of the p21 gene promoter containing three p53 binding sites, were PCR-amplified and cloned upstream of the HSV-1*tk* minimal promoter to form the luciferase reporter constructs p21intAHRELUC and p21promP53LUC, respectively. Diagramatic representation of these luciferase reporters bearing segments of the *PSA* and p21 promoters is shown in Fig. 1. The AHR and ARNT expression plasmids pcDNAI/B6AHR and mARNT have been described previously (Chang and Puga, 1998). pCMVp53, an expression plasmid for p53, was a generous gift from E. Knudsen. pCMVβgal (BD Biosciences Clontech) was cotransfected as a control to normalize transfection results for transfection efficiency. Empty pCDNAI or pBluescript II vectors were used to control for the total amount of DNA in each transfection.

Cell Proliferation Assays. Exponentially growing LNCaP cells, grown in IMEM containing 10% heat-inactivated complete FBS, were harvested and seeded in duplicate at 1×10^5 cells/well in poly-L-lysine–coated six-well plates. Poly-L-lysine (Sigma Chemical, St. Louis, MO) was used to promote cell adherence and had no effect on the growth rate of LNCaP cells (Knudsen et al., 1998). Cells were grown in IMEM with 5% CDT, 5% CDT + 100 pM R1881, 5% CDT + 10 nM TCDD, or 5% CDT + 100 pM R1881 + 10 nM TCDD over a 5-day period with treatments, and media were replenished every 2 days. Cells were harvested at 1, 3, and 5 days, and cell counts were determined using Trypan blue exclusion. All experimental points were done in duplicate, and experiments were repeated at least twice.

Bivariate Flow Cytometry. Cells were seeded at a density of 1.0×10^6 cells per 10-cm dish and synchronized for 3 days in IMEM plus CDT serum before the addition of various doses of DHT in the presence or absence of 10 nM TCDD. Treatments were continued for

96 h and were replenished after 48 h, followed by labeling with Cell Proliferation Labeling Reagent (Amersham Biosciences Inc., Piscataway, NJ) for 5 to 6 h. Cells were then rinsed with PBS, trypsinized, fixed with ethanol, and processed to detect BrdU incorporation with the BrdU flow kit (Promega, Madison, WI) according to the manufacturer's instructions. Analysis was carried out using the ModFit software program (Verity Software House, Topsham, ME). Experiments were done at least in duplicate, with 10,000 forward-scatter gated events scored for each sample. The results shown are the mean ± S.D. of each set of measurements.

DNA Content Determinations. Exponentially growing LNCaP cells were seeded at 2×10^4 cells/well in poly-L-lysine–coated six-well plates in 5% CDT FBS and allowed to attach for 24 h before treatment. Cells were treated with 0.1% dimethyl sulfoxide vehicle, 10 nM TCDD, 30 pM R1881, or 30 pM R1881 + 10 nM TCDD for 5 days. After treatment, cells were rinsed with 1× PBS and fixed with 75% EtOH at −20°C for 1 h. Fixed cells were incubated with 5 μg/ml Hoechst 33258 at room temperature in the dark for 10 to 30 min. Fluorescence was read in a Wallac Victor^2 1420 plate reader (PerkinElmer Wallac, Gaithersburg, MD) using 355-nm excitation and 460-nm emission wavelengths. Cell numbers were determined from a standard curve relating DNA content as measured by Hoescht 3358 fluorescence to cell number. Data are presented as mean ± S.D.

Real-Time PCR. Total RNA was isolated using TriReagent (MRC, Inc., Cincinnati, OH) according to the manufacturer's instructions with additional purification steps. cDNA was synthesized by reverse transcription of 20 μg of total RNA in a total volume of 30 μl containing 1× reverse transcriptase buffer, 2.5 μM random hexamers, 0.25 mM dNTP, 0.01 M dithiothreitol, 20 units of RNasin, and 200 units of SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). Samples were incubated at 42°C for 1 h. Residual RNA was degraded with 0.1 N NaOH and heating sample to 70°C for 10 min. For real-time PCR amplification of Hepa-1 mRNA, 1 μl of cDNA was amplified with mouse *Cyp1a1* primers (forward primer, 5′-GCCTTCATTCTGGAGACCTTCC-3′; reverse primer, 5′-CAATGGTCTCTCCGATGC-3′), giving a product of 280 bp between exons 5 and 7. For amplification of LNCaP mRNA, a similar quantity of cDNA was amplified with primers for human *Cyp1a1* (forward primer, 5′-CCTTCCGACACTCTTCCTTC-3′; reverse primer, 5′-AATCACCTTCTCACTTAACACC-3′), giving a 200-bp product in the corresponding region of the human *Cyp1a1* gene. β-Actin amplification of the same cDNA samples was used as an internal standard. Amplification was conducted in the Smart Cycler (Cepheid, Sunnyvale, CA) in a total volume of 25 μl consisting of 1× Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and 0.2 μM mouse Cyp1a1 primers. The reaction mixtures were heated to 95°C for 10 min and immediately cycled 40 times through a 24-s denaturing step at 95°C, a 60-s annealing step at 55°C, and a 46-s elongation step at 72°C. Cycle

threshold (C_T) of each sample was automatically determined to be the first cycle at which a significant increase in optical signal higher than an arbitrary baseline set at 30 fluorescence units was detected. All determinations were done in duplicate. The values shown represent the C_T ratios of experimental-to-control cells treated with Me$_2$SO, normalized to the β-actin mRNA level in the same sample.

Transfections and Reporter Assays. For transfection experiments, cells were plated in 24-well plates at a density of 1×10^5 cells/well and transfected at 70 to 80% confluence using LipofectAMINE Plus (Invitrogen). In brief, luciferase reporter constructs varying in amount from 100 ng to 3 μg, 100 ng of each expression vector for human AHR, ARNT, and β-galactosidase, and 500 ng of expression vectors for wild-type or mutant AR were incubated with Plus reagent for 15 min in serum- and antibiotic-free medium. LipofectAMINE was added, and the mixture was incubated for an additional 15 min. The transfection was carried out for 3 h, and thereafter the medium was changed to culture medium containing 5% CDT. Transfected cells were allowed to recover overnight, followed by treatment with DHT, TCDD, or R1881 at the concentrations indicated in the figures for a period of 24 or 48 h. After treatment, cells were washed twice with PBS and lysed with 100 μl of reporter lysis buffer (Promega). Aliquots (50 μl) of cell lysate were used to measure luciferase activity. Light units were determined immediately upon the addition of 150 μl of luciferase assay buffer (20 mM tricine, 1.07 mM MgCO$_3$, 2.67 mM MgSO$_4$, 33.3 mM dithiothreitol, 14.8 mg of coenzyme A, 530 μM ATP, 0.1 mM EDTA, and 10 mg of luciferin), using a Wallac Victor^2 1420 plate reader. β-Galactosidase activity was used to normalize luciferase measurements for transfection efficiency. For experiments to characterize the role of p53 in TCDD-induced p21 expression, cells were cotransfected with pCMVp53 and treated after transfection with 10 nM TCDD or vehicle for 36 h.

Western Blots. LNCaP cells were propagated in 5% CDT for 72 h before treatment with either 5% CDT, 0.1 nM DHT, 10 nM TCDD, or 0.1 nM DHT + 10 nM TCDD for 24 and 48 h. Cells were pelleted and lysed in a 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40 solution containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate. Lysates were subjected to brief sonication and clarified by centrifugation. For immunoblots, 50 μg of whole-cell lysate was loaded and subjected to SDS-PAGE. After electophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for blotting. The membrane was incubated with BLOTTO (5% fat-free milk powder in 1× PBS + 0.1% Tween 20, pH 7.5) for 2 h at room temperature and then incubated with primary antibodies to cyclin D1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p21$^{WAF1/CIP1}$ and p27^{KIP1} (Santa Cruz Biotechnology), RB (a gift from E. Knudsen), AHR (BIOMOL Research Laboratories, Plymouth Meeting, PA), and β-actin (a gift from J.
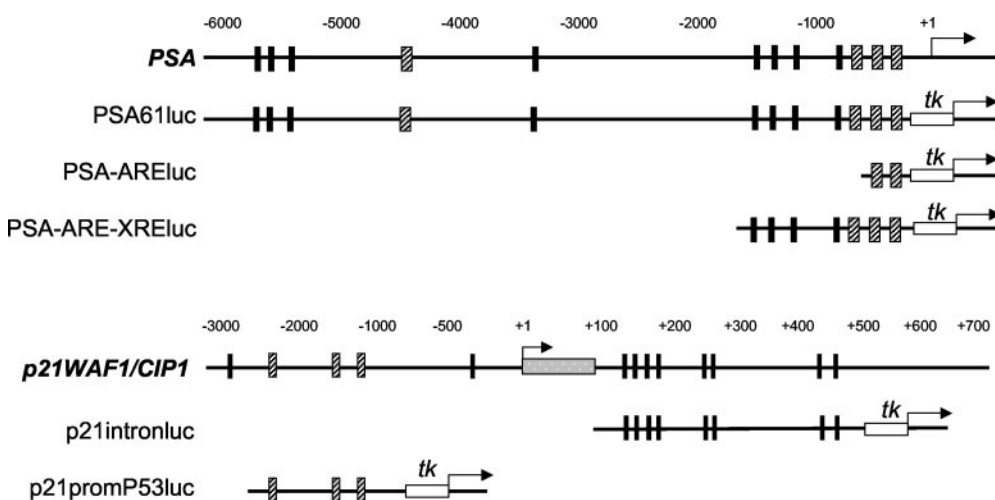

Fig. 1. Schematic representation of *PSA* and p21$^{CIP1/WAF1}$ regulatory sequences and luciferase reporters derived therein. Top, the upstream regulatory domain of the human *PSA* gene from coordinates −6000 to the transcriptional start site at +1. ■, AHRE motif; ▨, ARE motif. Bottom, not drawn to scale, shows the architecture of the human p21$^{CIP1/WAF1}$ promoter, first exon, and part of the first intron from coordinates −3000 to +700. The first exon is represented by the stippled box between coordinates +1 and +90. ■, AHRE motif; ▨, p53-responsive sites.

Lessard). The blot was washed three times with $1\times$ PBS + 0.1% Tween 20, pH 7.5, incubated with a 1:10,000 dilution of secondary antibody for 1 h, and developed with the ECL detection system (Amersham Biosciences).

Statistical Analyses. Group comparisons were made by one-way analysis of variance. Differences were considered significant at $p < 0.05$.

Results

TCDD Inhibits DHT-Induced Cell Proliferation in LNCaP Cells. Growth and maintenance of the prostate is dependent on serum androgen. LNCaP cells, unlike androgen-independent prostate cancer cell lines, have retained this androgen requirement for proliferation and have been used extensively in prostate cancer research and in the development of anticancer drugs. In prostate cells, testosterone is converted to DHT, the major AR agonist responsible for regulation of gene expression and cell growth. To test the effect of TCDD on AR-mediated functions, we used exponentially growing LNCaP cells stimulated by R1881, a nonmetabolizable DHT analog. Cells grown in 5% steroid-depleted CDT showed less than a 50% increase over the 5-day duration of the experiment, in agreement with previous studies (Knudsen et al., 1998), attesting to the androgen requirement of these cells for growth. Supplementation of CDT with 100 pM R1881 restored growth; the cells exhibited a doubling time of 3 days. In contrast, TCDD blocked the restoration of

growth by R1881 and maintained the cells in a growth-arrested state (Fig. 2A). Cell viability, measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, was not decreased in response to TCDD treatment alone or in cotreatment with DHT (data not shown). As an estimate of S-phase entry, bivariate flow cytometry analysis was conducted after BrdU labeling of LNCaP cells synchronized by culturing in CDT serum for 3 days before treatment with increased concentrations of DHT in the presence or absence of TCDD. TCDD decreased the percentage of cells positive for BrdU at each dose of DHT tested (Fig. 2B). To confirm the effect of TCDD on DNA synthesis, overall DNA content was measured by Hoescht 3358 staining of cells treated with TCDD after induction with R1881. TCDD was found to reduce the Hoescht fluorescence by approximately 1.7-fold (Fig. 2C). These data indicated that TCDD treatment blocks androgen-dependent proliferation of LNCaP cells.

TCDD Blocks Cyclin D1 Expression and DHT-Mediated Phosphorylation of RB. It has been shown that the growth response of LNCaP cells to androgen stimulation depends on the phosphorylation state of the RB protein (Knudsen et al., 1998). To characterize the effect of TCDD on androgen-dependent cell-cycle progression, Western blot analyses of whole-cell extracts from synchronized LNCaP cells were subjected to SDS-PAGE and RB phosphorylation status, and expression levels of cyclin D1 and p21 were evaluated. Cells maintained in 5% steroid-depleted medium
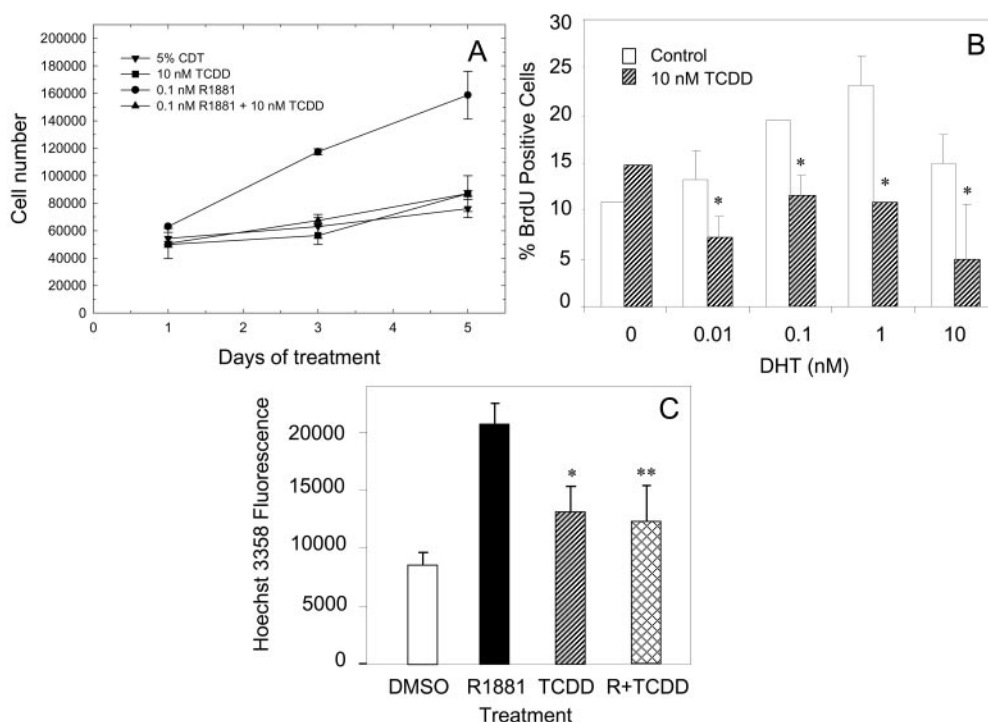

Fig. 2. Inhibition of androgen-dependent cell proliferation in LNCaP cells by TCDD. A, cells grown in IMEM supplemented with 10% complete serum were seeded at 1×10^5 cells/well in poly-L-lysine–coated six-well plates in either 5% CDT medium with or without 10 nM TCDD or 5% CDT medium + 100 pM R1881 with or without 10 nM TCDD. Cells were harvested at 1, 3, and 5 days and counts were determined using Trypan blue exclusion. B, cells were seeded at a density of 1.0×10^6 cells per 10-cm dish and synchronized for 3 days in CDT serum before incubating with increasing doses of DHT in the presence or absence of 10 nM TCDD. Treatments were continued for 96 h and were replenished after 48 h, followed by labeling with Cell Proliferation Labeling Reagent (Amersham Biosciences) for 5 to 6 h. Cells were processed to detect BrdU incorporation with the BrdU flow kit (Promega) according to manufacturer's instructions and analyzed by fluorescence-activated cell sorting. C, 2×10^4 cells/well were seeded in poly-L-lysine–coated six-well plates in 5% steroid-depleted fetal bovine serum (CDT) and allowed to attach for 24 h before treatment with vehicle (control) with or without 10 nM TCDD and 0.03 nM R1881 with or without 10 nM TCDD for 5 days. Cell numbers were determined as a measure of DNA content (Hoescht 3358 fluorescence). Experiments were done at least in duplicate with 100 events scored per sample. Means ± S.D. are represented for each experiment. ⋆, significant differences from control at $p < 0.05$.

showed low levels of cyclin D1 and p21^{WAF1CIP1} expression and low or minimal RB phosphorylation (Fig. 3). Consistent with other reports (Knudsen et al., 1999a,b), treatment with DHT stimulated phosphorylation of RB and increased the expression levels of cyclin D1 and p21 after treatment for 24 or 48 h (Fig. 3). TCDD treatment did not change the expression levels of cyclin D1 or the phosphorylation status of RB, which was similar to the background levels seen in control cells at 24 h; however, p21 expression was induced in response to TCDD treatment alone but was repressed when cells were treated with TCDD in the presence of DHT. Likewise, DHT-induced RB phosphorylation was inhibited by TCDD treatment (Fig. 3). These results suggest that TCDD blocks the proliferative effect of DHT on expression and activity of proteins required for the cell cycle to progress and inhibits the androgen-dependent phosphorylation of RB.

TCDD Induces Expression of p21 Independently of p53. p21$^{WAF1/CIP1}$ blocks cell-cycle progression by binding cyclin D1/cdk-4 and cyclin E/cdk-2 complexes and inhibiting their kinase activities, leading to the hypophosphorylation of RB. The tumor suppressor p53 activates p21$^{WAF1/CIP1}$ expression, causing cell-cycle arrest. Computer scanning of the promoter and first intron of the p21$^{WAF1/CIP1}$ gene reveals the presence of seven complete AhREs, also found in p16^{INK4A}, another cyclin-dependent kinase inhibitor. These AhREs could serve as AHR-dependent enhancers to increase p21 or p16 gene expression. On the other hand, the effect of TCDD on p21 expression could be a secondary effect of p53 up-regulation or stabilization. To test whether these potential AHR-ARNT binding regions were responsive to TCDD treatment, a 399-bp fragment of the first intron of the human p21 gene containing seven AhRE sites was PCR-amplified and cloned upstream of the HSV-1tk minimal promoter and luciferase reporter to form the luciferase reporter construct p21intAHRELUC. LNCaP and MCF-7 cells were transiently transfected with fixed amounts of p21intAHRLUC and pCMVβgal. Transfections were done also with added expression vectors for AHR and ARNT to enhance the effects, if any, of the AHR complex. In the absence of AHR and ARNT, neither cell line showed detectable luciferase activity in response to TCDD treatment; however, in cells overexpressing AHR and ARNT, TCDD treatment caused 40- and 20-fold induction in luciferase activity over controls in LNCaP and

MCF-7 cells, respectively (Fig. 4A). Thus, the first intron of the p21$^{WAF1/CIP1}$ gene contains regulatory elements that are highly inducible by TCDD. To determine whether p53 had an additional role in TCDD-induced p21 enhancer activity, a 1.3-kb fragment of the p21 gene containing three p53 binding sites was PCR-amplified and cloned upstream of the HSV-1tk minimal promoter and luciferase gene to form the luciferase reporter construct p21promP53LUC. LNCaP and MCF-7 cells were transiently transfected with this plasmid plus equimolar concentrations of plasmids expressing AHR and ARNT and pCMVp53, an expression vector for human p53. In both LNCaP and MCF-7 cells, transfection of pCMVp53 increased significantly the luciferase levels of the p53-responsive reporter; however, neither the presence of AHR and ARNT, of TCDD, or of both changed significantly the luciferase levels resulting from p53 expression (Fig. 4B). These results show that TCDD does not require the p53 pathway to induce p21 expression and that induction of p21 is most probably a direct transcriptional effect of the activated AHR.

Activated AHR Blocks AR-Induced Transcriptional Activity from Promoters Derived from Various Androgen-Responsive Genes. Although LNCaP cells express both AHR and ARNT proteins, they express significantly lower levels of AHR compared with Hepa-1 cells or mouse embryo fibroblasts from AHR+/+ mice (Fig. 5A). At the mRNA level, LNCaP cells respond to TCDD by inducing a 40-fold increase in CYP1A1 mRNA, almost as robust as the 120-fold response seen in mouse hepatoma Hepa-1 cells (Fig. 5B); however, the amount relative to β-actin of induced CYP1A1 mRNA in LNCaP cells is approximately 1400 times lower than in Hepa-1 cells (Fig. 5C), suggesting that, in overall terms of AHR-mediated transcriptional activity, LNCaP cells are significantly less responsive to TCDD than Hepa-1 cells, the standard cell line in the study of TCDD effects. Notwithstanding, TCDD has been found to decrease androgen-induced PSA protein and mRNA levels in LNCaP cells (Jana et al., 1999), suggesting that TCDD might also disrupt AR transcriptional activity. To address this question under conditions of greater AHR-dependent transcriptional responses, we used AR-driven reporters in AR-expressing LNCaP cells and AR-nonexpressing CV-1 cells and tested whether TCDD blocked androgen-dependent reporter expression. LNCaP cells were transfected with PSA61luc, which
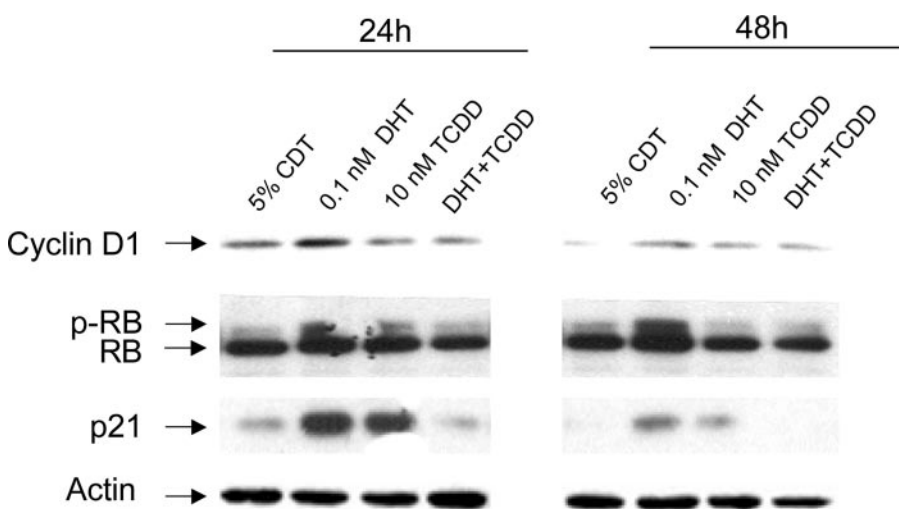

Fig. 3. DHT-induced cyclin D1 expression and RB phosphorylation are decreased in the presence of TCDD. LNCaP cells were propagated in 5% CDT for 72 h before treatment with either 5% CDT, 0.1 nM DHT, 10 nM TCDD, or 0.1 nM DHT + 10 nM TCDD for 24 and 48 h. Whole-cell extracts were obtained, and proteins were subjected to 7.5 and 12.5% SDS-PAGE followed by transfer to nitrocellulose membranes. Western immunoblots applying specific polyclonal antibodies were used in detecting protein levels of cyclin D1, hypophosphorylated RB, and p21$^{WAF1/CIP1}$.

contains three androgen-responsive elements (ARE I, II, and III) in a 6.1-kb stretch of DNA isolated from the human *PSA* gene promoter and ARR2PBluc, which contains two AR-responsive regions. AREs I and II are located in the promoter region, and ARE III is located further upstream in the enhancer region. In the absence of the androgen analog R1881, PSA61luc directs the expression of very low basal levels of luciferase, which are stimulated approximately 30-fold by the addition of R1881 (Fig. 6A).

TCDD, alone or in the presence of R1881, had no significant effect on luciferase levels. However, when AHR and ARNT were overexpressed, there was a dramatic decrease in R1881-induced luciferase activity even in the absence of TCDD, suggesting that the presence of high levels of AHR-ARNT is sufficient to down-regulate AR function. A similar inhibitory effect was observed when ARR2PBluc was used as the readout (Fig. 6B), indicating that the inhibitory effect of activated AHR is not promoter-specific.
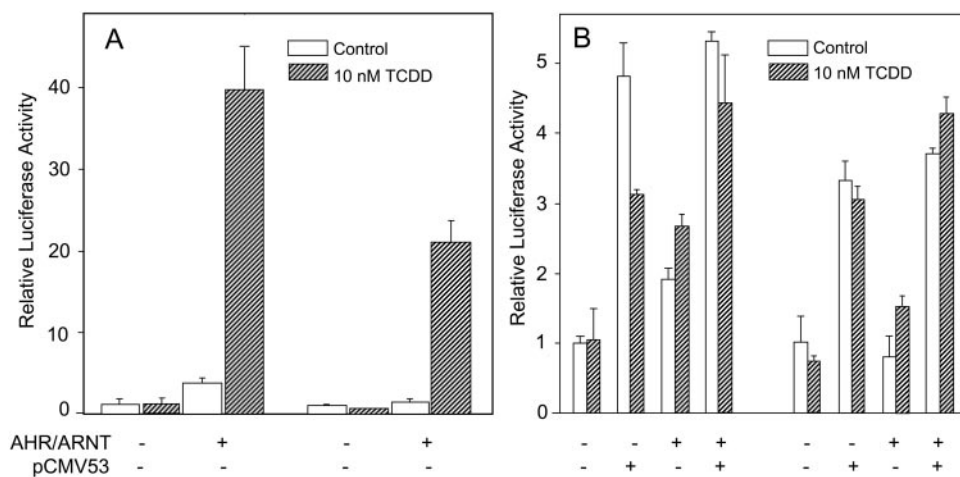

Fig. 4. TCDD induces reporter luciferase activity through AhRE enhancer sequences in the p21 first intron. A 399-bp fragment of the first intron of the human p21 gene containing seven complete AhRE motifs and a 1.3-kb fragment of the p21 promoter containing three p53-responsive regions were PCR-amplified and cloned upstream of the HSV-1*tk* minimal promoter to form two distinct luciferase reporter constructs, p21intAHRELUC and p21promP53LUC. LNCaP and MCF-7 cells were transiently transfected with either the p21intAHRLUC (Fig. 4A) or the p21promP53LUC (Fig. 4B) reporter constructs and pCMVβgal with and without equimolar amounts of plasmids expressing AHR and ARNT. Cells were treated with 10 nM TCDD for 24 h, and luciferase activity was determined. To characterize the role of p53 in TCDD-induced p21 expression, cells were cotransfected with pCMVp53 (Fig. 4B) followed by treatment with 10 nM TCDD after transfection for 36 h. Luciferase and β-galactosidase activities were measured as described under *Materials and Methods*. Luciferase values were normalized to β-galactosidase activities and are represented as mean ± S.D.
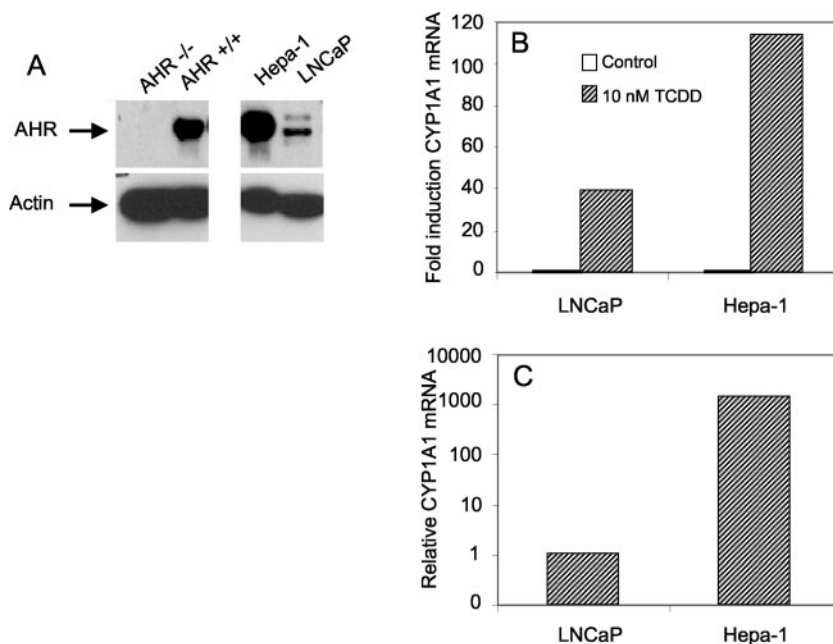

Fig. 5. LNCaP cells express low levels of AHR and are less responsive to CYP1A1 mRNA induction by TCDD than Hepa-1 cells. A, LNCaP cells were cultured in 5% CDT-containing medium for 3 days before treatment with either 0.1% vehicle or 10 nM TCDD for either 2 or 4 h, as indicated. Whole-cell extracts were separated by SDS-PAGE on a 7.5% acrylamide gel. Antibodies to AHR and β-actin were used and visualized with the ECL kit and autoradiography. Of the two bands shown in the lane labeled LNCaP, the lower corresponds to the human AHR protein, and the upper is a cross-reactive protein that binds the anti-mouse AHR antibodies used in this experiments. B, RNA was extracted and reverse-transcribed to cDNA from mouse Hepa-1 and LNCaP cells treated with 0.1% vehicle or 10 nM TCDD. cDNA was PCR-amplified using the Light Cycle Real-Time PCR and SYBR Green. C, the ratio of fold induction determined from the C_T values for CYP1A1 and β-actin mRNAs are represented for both cell lines. The β-actin mRNA levels were similar for the two cell lines.

A scan of the 6.1-kb fragment of the human *PSA* gene regulatory region used in the PSA61luc construct revealed the presence of AhREs that may act as transcriptional inhibitory motifs (Saatcioglu et al., 1990; Zacharewski et al., 1994; Kharat and Saatcioglu, 1996). We found eight sites, located at the 5′ end of the regulatory region of the *PSA* gene and distal to AREs I, II, and III, that might interact with the AHR-ARNT binding complex. This allowed for separation of the AR-responsive region from the potential AHR-responsive sequences of the *PSA* gene promoter. We used restriction enzymes to isolate the AR- and AHR-responsive fragments and ligated these fragments into the promoterless PGL3Basic reporter to construct PSA-AREluc and PSA-ARE-AhREluc, respectively. LNCaP cells could not be used for these functional studies because they require the presence of the AREIII enhancer sequences for maximal induction (data not shown), which seems to be a characteristic of LNCaP cells already observed by others (Cleutjens et al., 1997). Instead, we used CV-1 cells, which do not express AHR or AR proteins but do express ARNT. These cells are also characterized by the constitutive activation of ectopically expressed AHR, possibly because of the presence of an endogenous AHR ligand (Chang and Puga, 1998). Neither full-length nor variant PSA constructs responded to DHT in the absence of AR, which confirms the absence of endogenous AR expression in these cells. Both PSA-variant reporter constructs showed higher basal and induced levels of activity compared with PSA61luc, which suggests the presence of inhibitory sequences between the enhancer and promoter regions of *PSA* (Fig. 7, A–C). All three reporters responded equally well to DHT in the presence or absence of TCDD; however, both basal and induced AR-driven activity was significantly reduced when AHR was expressed alone or when cotransfected with ARNT. CV-1 cells express high levels of ARNT, which explains why additional ARNT had no significant effect on the reduction of AR transcriptional activity. Hence, AHR expression is required for the inhibitory effect of TCDD on AR-driven gene transcription, as observed in studies with AHR knockout mice (Lin et al., 2002). The presence of the activated AHR blocked AR-driven reporter activity from all three reporters regardless of the presence of AhRE motifs in the regulatory elements of the promoter, indicating that AHR directly inhibits AR activity through squelching mechanisms independent of the binding to inhibitory motifs in the regulatory region of the *PSA* gene.

Discussion

The data presented here show that TCDD blocks androgen-dependent cell proliferation in LNCaP cells without loss of viability, indicating that TCDD does not induce apoptosis in these cells. Decreases in BrdU uptake and DNA content strongly suggest that TCDD arrests cells before S-phase, presumably in G_1. Analyses of the expression levels of regulatory proteins controlling the G_1 to S phase cell-cycle progression showed that TCDD blocks androgen-induced hyperphosphorylation of RB, reduces the level of cyclin D1, and induces the expression of p21. In addition, transient transfection studies showed that TCDD directly regulates p21 gene expression independently of p53. LNCaP cells were considerably less responsive to TCDD-dependent *CYP1A1* induction than Hepa-1 or mouse embryo fibroblasts cells, possibly because of a much lower level of AHR expression. In addition, overexpression of AHR attenuated AR-mediated gene transcription independently of cell type.

Cell-cycle progression is tightly controlled by positive and negative regulators, such as the cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, and the tumor suppressors, such as p53 and RB. One of the key regulatory events governing cell-cycle progression past the G_1 checkpoint is the phosphorylation state of RB, which is modulated by the kinase activities of cyclin/cdk complexes. During G_0 and early-to-mid G_1 phases, cyclins D1 and E complex with cdks-4 and -2, respectively, to phosphorylate RB, which in its unphosphorylated state binds to and inactivates E2F, a transcription factor that regulates the expression of S-phase promoting genes (Cheng et al., 1999; Sherr and Roberts, 1999). Cyclin-dependent kinase inhibitors, such as p21$^{WAF1/CIP1}$ and p27^{KIP1}, bind to cyclin/cdk complexes and block their catalytic activities, thereby causing an inhibitory effect on cell-cycle progression by maintaining the levels of hypophosphorylated RB (Coqueret, 2003).
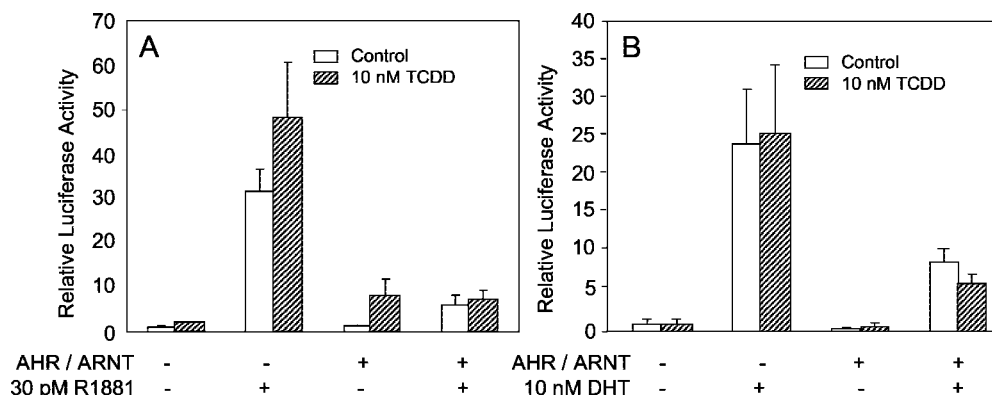

Fig. 6. Overexpression of AHR inhibits AR-mediated *PSA* gene promoter activity. LNCaP cells were cultured in phenol-red free CDT medium containing growth factors but devoid of steroids. After 24 h, cells were cotransfected with either 3.0 μg of PSA61luc (A) or 250 ng of PBARR2luc (B), containing a luciferase reporter fused to the promoter of the human prostate-specific antigen or to the promoter of rat probasin gene, respectively, along with 100 ng of pCMVhuAHR, 100 ng of pCMVhuARNT, 100 ng of pCMVβgal, and pCDNAI empty vector when needed to complete an equimolar mass of DNA for each transfection. After transfection, cells were treated with 0.1% vehicle or with the indicated androgen in the absence (▨) or presence (■) of 10 nM TCDD for 48 h. Luciferase and β-galactosidase activities were measured as described under *Materials and Methods*. Luciferase values were normalized to β-galactosidase activities and are presented as the mean ± S.D.

Numerous studies have shown cell-cycle–dependent effects of TCDD on gene expression and AHR-dependent and -independent effects of TCDD on cell-cycle progression (Gottlicher et al., 1990; Weiss et al., 1996; Wang et al., 1998). Expression of the immediate early genes, c-*Fos* and c-*Jun*, and subsequent increase in the transcriptional activity of AP-1 are induced by TCDD (Puga et al., 1992; Hoffer et al., 1996). AHR indirectly induces c-*Myc* expression through an AHR/RelA DNA-binding complex that binds to the nuclear factor-κB–responsive element in the c-*Myc* gene promoter of human breast cancer cells (Abbott et al., 1999). Other studies have shown physiological roles of the activated AHR in cell-cycle regulation, signal transduction, and apoptosis through direct association with key cell-cycle regulatory proteins such as RB (Ge and Elferink, 1998; Puga et al., 2000; Strobeck et al., 2000) that repress cell-cycle progression in murine hepatoma cells. TCDD also blocks cell proliferation in rat 5L hepatoma cells and fetal thymocytes by inducing expression of the cyclin-dependent kinase inhibitor p27^{KIP1} (Ge and Elferink, 1998; Kolluri et al., 1999). In estrogen-responsive MCF-7 cells, TCDD blocks G_1 to S progression, concomitantly with decreases in estrogen-induced cyclin D1, cdk-4, cdk-2, and RB phosphorylation and increases in p21$^{WAF1/CIP1}$ protein levels (Wang et al., 1998).

Our findings that TCDD blocks DHT-induced cell proliferation in LNCaP cells are in good agreement with data published previously by others (Jana et al., 1999). We have extended that work to the molecular level and characterized some of the mechanisms responsible for cross-talk of AHR- and AR-mediated pathways. Androgens and estrogens induce cell-cycle progression and regulate gene expression by very similar molecular mechanisms. Given the antiproliferative effects of TCDD in the reproductive systems of male and female rodents and in estrogen-responsive MCF-7 breast cancer cells, it seemed likely that TCDD would interfere with androgenic functions through mechanism(s) similar to those responsive for the inhibition of estrogenic responses. Indeed, as it was shown for estrogenic responses in MCF-7 cells (Wang et al., 1998), TCDD-induced growth arrest of LNCaP cells involved the modulation of multiple cell-cycle regulatory proteins that resulted in hypophosphorylation of RB. AHR has been found to bind preferably to hypophosphorylated RB and to act synergistically with RB to repress the transcriptional activity of E2F-DP1 dimers (Ge and Elferink, 1998; Puga et al., 2000; Strobeck et al., 2000), suggesting that TCDD might block androgen-induced proliferation of LNCaP cells by stimulating the interaction between AHR and RB. Interactions between AHR and RB might compete with interactions between AR and RB, reported by others to promote AR-dependent transcriptional activity (Lu and Danielsen, 1998; Yeh et al., 1998). On the other hand, decreases in cyclin D1 and increases in p21 expression suggest an additional mechanism whereby TCDD might modulate the kinase activity of the cyclin/cdk complexes that phosphorylate RB, as seen in MCF-7 cells.

The p21$^{WAF1/CIP1}$ protein plays at least three separate roles during the cell cycle (Coqueret, 2003). The first is a p53-dependent function involving the arrest of damaged cells before the onset of DNA synthesis. The second is a p53-independent role in the assembly and stabilization of the cyclin D1-cdk-4 complexes that promote RB phosphorylation during G_1. The third is the inhibition of cell-cycle progression through binding to cyclin E-cdk-2 complexes and blocking their associated kinase activity when the level of p21 surpasses a threshold level. We find that TCDD treatment increases p21 expression in LNCaP cells independently of p53,
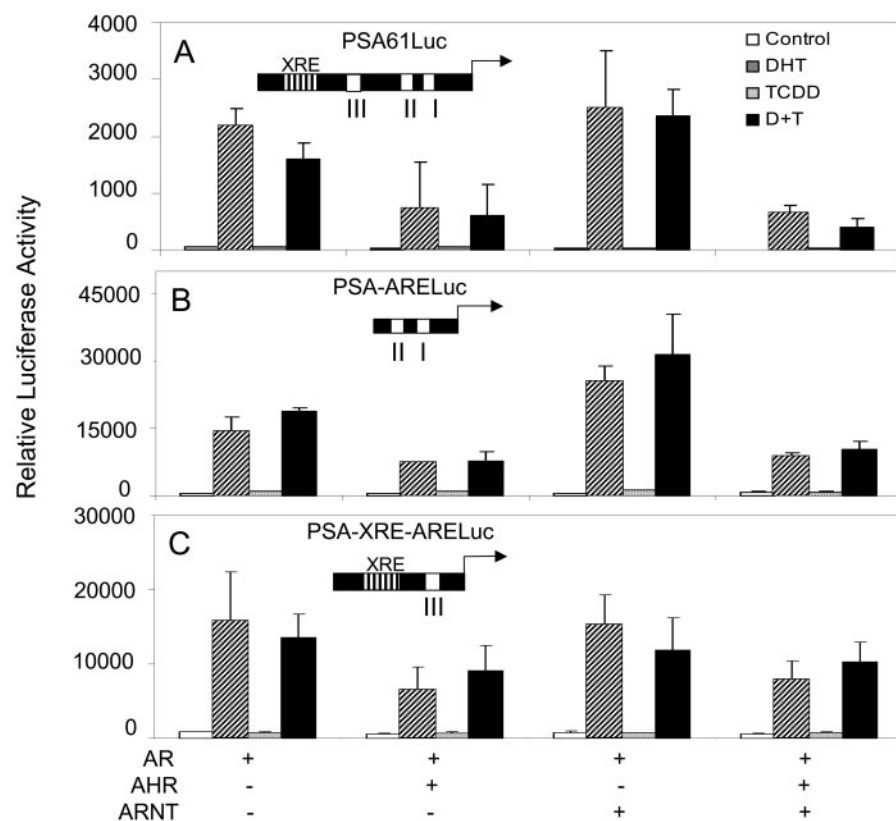
Fig. 7. AHR inhibits AR-mediated *PSA* promoter activity through AREs and not through binding AhREs. CV-1 cells were transiently transfected with 100 ng of each of the reporter constructs indicated, along with the expression vectors for human AHR, ARNT, β-galactosidase, and 500 ng of the expression vectors for wild-type AR. Cells were allowed to recover for 16 h, followed by treatment with DHT, TCDD, or both for 24 h. Luciferase values were normalized to β-galactosidase activities and are represented as the mean ± S.D.

suggesting that TCDD may initially function as a positive regulator of cell-cycle progression by promoting the assembly of cyclin D1-cdk-4 complexes but may ultimately regulate progression negatively by inhibiting the kinase activity of cyclin E-cdk-2 complexes and the phosphorylation of RB. In agreement with previous findings {Knudsen et al., 1998), DHT treatment induces p21 and cyclin D1 expression and blocks the phosphorylation of RB. Even though both TCDD and DHT increase p21 expression levels, combined treatment of LNCaP cells with TCDD and DHT results paradoxically in the reduction of p21 to nearly undetectable levels. Low levels of p21 do not effectively promote cyclin D1-cdk-4 complex formation and, although for different causes than p21 over-expression, might lead to the same ultimate effect, namely RB hypophosphorylation and cell-cycle arrest. At present, we have no molecular explanation for the differences in p21 expression observed between TCDD and TCDD + DHT treatments. On the other hand, other cyclin-dependent kinase inhibitors, such as p16^{INK4A}, which also has AHR-response elements in its promoter, may be involved in the inhibition of RB phosphorylation and, ultimately, in cell-cycle arrest. A similar effect might also result from the reduced levels of cyclin D1 in cells exposed to TCDD + DHT compared with DHT alone. Lower levels of cyclin D1 will decrease the levels of functional cyclin D1/cdk-4 complexes and the phosphorylation of RB. It is likely that all of these mechanisms act combinatorially and that the ultimate effects, RB hypophosphorylation and cell-cycle arrest, result from their concerted action.

Cyclin D1 expression is the key rate-limiting step in the formation of cyclin D1-cdk-4 complexes. Cyclin D1 is positively regulated by mitogenic factors that increase AP-1 activity, Ras transformation, and ectopic expression of c-*Jun* (Bakiri et al., 2000). DHT induces cyclin D1 expression in LNCaP cells, an effect that is blocked by TCDD (Knudsen et al., 1998) (Fig. 3). TCDD blocks estradiol, transforming growth factor-α, epidermal growth factor, insulin-like growth factor-I, and insulin-stimulated growth in breast cancer cells (Fernandez and Safe, 1992), and it is likely that it might also modulate the growth factor signals which regulate the expression of cyclin D1. Consistent with this idea, we find that the activated AHR blocks AR-mediated transcriptional activity of several AR-responsive reporters, including PSA and probasin. Hence, it seems that the inhibition of androgen-induced cell-cycle progression in LNCaP cells by TCDD takes place through a mechanism that blocks the AR-mediated increase of cell-cycle regulatory proteins and leads to the hypophosphorylation of RB.

Acknowledgments

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