Molecular Pharmacology Fast Forward. Published on May 26, 2004 as doi: 10.1124/mol.104.000356
This article has been copyedited and formatted. The final version may differ from this version.

TCDD blocks androgen-dependent cell proliferation of LNCaP cells through modulation of RB phosphorylation*

Sonya Barnes-Ellerbe, Karen E. Knudsen,and Alvaro Puga

Center for Environmental Genetics

Department of Environmental Health (S.B.-E. and A.P.)

and

Department of Cell Biology (K.E.K.)

University of Cincinnati College of Medicine

Cincinnati, OH 45221

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: TCDD blocks androgen-dependent proliferation of LNCaP cells

Corresponding author:

Alvaro Puga

Department of Environmental Health, University of Cincinnati Medical Center

P.O. Box 670056. Cincinnati, OH 45367-00567

(Messenger Mail, use: 123 E. Shields St. Cincinnati, OH 45220)

Phone: (513) 558-0916; FAX: (513) 558-0925

E-mail: Alvaro.Puga@UC.EDU

This manuscript contains 35 text pages

7 figures

40 references.

Abstract: 217 words.

Introduction: 790 words.

Discussion: 1,240 words.

Abreviations :

AHR: Aryl hydrocarbon receptor; AhRE: Ah receptor response element (also named XRE, for xenobiotic, and DRE, for dioxin response element); AR: Androgen receptor; ARE: Androgen receptor response element; ARNT: Aryl hydrocarbon receptor nuclear translocator; bHLH: basic region-helix-loop-helix; CDT: Charcoal-dextran treated FBS; DHFR: Dihydrofolate reductase; DHT: Dihydrotestosterone; ER: Estrogen receptor; FBS: Fetal bovine serum; HSP: Heat-shock protein; IMEM: Improved minimal essential médium; LNCaP: Lymph Node Cancer of the Prostate; NQO-1: NAD(P)H-dependent quinone oxidoreductase-1; PAS: PER-ARNT-SIM; PSA: Prostate-specific antigen; RB: Retinoblastoma; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

ABSTRACT

Cell cycle regulatory events associated with inhibition of androgen-dependent cell proliferation by TCDD were studied in the human-derived LNCaP cell line. TCDD blocked the G1-S transition of LNCaP cells synchronized in G0/G1 when these cells were induced to reinitiate cell cycle progression by DHT. Western blot analyses of these cells revealed altered expression levels of G1 regulatory proteins, including increases in hypophosphorylated RB protein and concomitant decreases in cyclin D1. $p21^{WAF1/CIP1}$, which is involved in the assembly of cyclin D1/cdk-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 AHR 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 of androgen receptor-mediated gene transcription through receptor cross-talk, possibly involving competition for coregulators or by direct protein interaction.

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

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. Briefly, TCDD binds to the AHR and facilitates the release of bound HSP90 proteins resulting in receptor activation. Subsequent to this release, liganded AHR translocates to the nucleus where it heterodimerizes with ARNT. AHR and ARNT are similar in structure and belong to the bHLH/PAS 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 NQO-1 and others (reviewed in 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 male accessory sex organs, imprinting responses of the accessory sex organs for later androgenic stimulation, initiating spermatogenesis, and for 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 similar pattern 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 HSP90 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 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;Roman et al., 1998), which suggests that TCDD does not alter the synthesis or metabolism of androgen to cause anti-androgenicity. More likely, TCDD acts directly on target organs to disrupt androgen-induced responses at the level of gene transcription, cell proliferation, or both.

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

Epidemiological studies in humans and i*n 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 the 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 anti-estrogen 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.

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Cell culture and treatments. LNCaP, Hepa- 1, CV-1, and MCF-7 cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD) and maintained in a 5% CO_2 atmosphere. For normal growth, LNCaP cells were maintained in phenol red-free IMEM (Biosource) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, and 100 units/ml penicillin/ streptomycin (Gibco). Hepa-1, MCF-7 and CV-1 cells were maintained in $\tilde{\alpha}$MEM supplemented with 5% FBS (Gibco) and 100 units/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 promoter-less luciferase reporter vector (Clonetech). PSA-AhRE-AREluc was constructed by digesting the full-length PSA61luc reporter construct with *HindIII* and *KpnI* and subcloning the 1.5kb fragment into the PGL3Basic reporter (Clonetech). ARR2PB-LUC is a reporter containing the androgen-responsive region of the rat probasin gene promoter; pSG5-AR is an 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 1.3kb 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

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Clonetech) was co-transfected 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 x 10^5 cells/well in poly-L-lysine coated 6-well plates. Poly-L-lysine (Sigma) 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 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 X 10^6 cells/10-cm dish and synchronized for 3 days in IMEM plus CDT serum before addition of various doses of DHT in the presence or absence of 10 nM TCDD. Treatments were continued for 96 hrs and were replenished after 48 hrs followed by labeling with Cell Proliferation Labeling Reagent (Amersham Pharmacia Biotech) for 5-6 hrs. Cells were then rinsed with PBS, trypsinized, fixed with ethanol, and processed to detect BrdU incorporation

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

with the BrdU flow kit (Promega, Madison, WI) according to the manufacturer's instructions. Analysis was carried out using the MODFit software program. Experiments were done at least in duplicate with 10,000 forward scatter gated events scored for each sample. The results shown are the mean ± SD of each set of measurements.

DNA content determinations. Exponentially growing LNCaP cells were seeded at 2 X 10^4 cells/well in poly-l-lysine coated 6-well plates in 5% CDT FBS and allowed to attach for 24 h prior to treatment. Cells were treated with 0.1% DMSO vehicle, 10 nM TCDD, 30 pM R1881 or 30 pM R1881 + 10 nM TCDD for 5 days. After treatment, cells were rinsed with 1X PBS and fixed with 75% ETOH at - 20ºC for one hour. Fixed cells were incubated with 5 µg/ml Hoechst 33258 at room temperature in the dark for 10-30 minutes. Fluorescence was read in a Wallac Victor^2 1420 plate reader, 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 ± SD.

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 1x 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^{TM} 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

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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 quantitity 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 1X Brilliant™ SYBR® Green QPCR Master Mix (Stratagene) 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 above an arbitrary base line 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_2SO, 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 x 10^5 cells/well and transfected at 70–80% confluence using Lipofectamine Plus (Invitrogen). Briefly, luciferase reporter constructs varying in amount from 100 ng to 3 µg, 100 ng each of expression vectors 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.

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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, Madison, WI). Aliquots of 50 µl of cell lysate were used to measure luciferase activity. Light units were determined immediately upon addition of 150 µl of luciferase assay buffer (20 mM Tricine, 1.07 mM $MgCO_2$, 2.67 mM $MgSO_4$, 33.3 mM dithiothreitol, 14.8 mg 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 co-transfected with pCMVp53 and treated post-transfection with 10 nM TCDD or vehicle for 36 h.

Western blots. LNCaP cells were propagated in 5% CDT for 72 h prior to 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 NETN (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) solution containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 mM sodium fluoride, 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. Following electophoresis, proteins were transferred to nitrocellulose membranes (BioRad) for blotting. The membrane was incubated with

BLOTTO (5% fat-free milk powder in PBST [1X Phosphate Buffer Saline + 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), $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Santa Cruz Biotechnology), RB (a gift from E. Knudsen), AHR (Biomol), and β-actin (a gift from J. Lessard). The blot was washed 3 times with PBST, incubated with a 1:10,000 dilution of secondary antibody for 1 h, and developed with the Amersham ECL detection system.

Statistical analyses

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

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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 anti-cancer 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 non-metabolizable 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, with the cells exhibiting 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 MTT assay, was not decreased in response to TCDD treatment alone or in co-treatment 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 prior to 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 about 1.7 fold (Fig.

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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 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;Knudsen et al., 1999b), 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 were induced in response to TCDD treatment alone but were repressed when cells were treated with TCDD in the presence of DHT; similarly, 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/cdk4 and cyclin E/cdk2 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. Alternatively, the effect of TCDD on p21 expression could be a secondary effect of p53 upregulation 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-1 *tk* 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 pCMV□gal. 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 if 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-1 *tk* 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

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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 most likely, induction of p21 is 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 when compared to Hepa-1 cells or to 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 1,400 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 to study 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 utilized AR-driven reporters in AR-expressing LNCaP cells and AR-non-expressing CV-1 cells and tested whether TCDD blocked androgen-dependent reporter expression. LNCaP cells were transfected with PSA61luc, which contains three androgen responsive elements (ARE I, II, and III) in a

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

6.1kb stretch of DNA isolated from the human prostate specific antigen (*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 by 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 over-expressed, 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.

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 might 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 utilized restriction enzymes to isolate the AR and AHR responsive fragments and ligated these fragments into the promoter-less 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 due to 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 to 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 co-transfected 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 prior to S-phase, presumably in G1. Analyses of the expression levels of regulatory proteins controlling G1-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 due to a much lower level of AHR expression. In addition, over-expression 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 cycle progression past the G1 checkpoint is the phosphorylation state of RB, which is modulated by the kinase activities of cyclin/cdk complexes. During G0 and early-mid G1 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 (Sherr and Roberts, 1999;Cheng et al., 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).

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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 NF-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 G1-S progression, concomitantly with decreases in estrogen-induced cyclin D1, cdk-4 and -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 previously published data 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 anti-proliferative effects of TCDD in the reproductive systems of male and female rodents and in estrogen-responsive MCF-7 breast cancer

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

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). Alternatively, 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-cdk4 complexes that promote RB phosphorylation during G1. The third is the inhibition of cell cycle progression through binding to cyclin E-cdk2 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, suggesting that TCDD may initially function as a positive regulator of cell cycle progression by promoting the assembly of cyclin D1-cdk4 complexes, but ultimately regulate progression negatively by inhibiting

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

the kinase activity of cyclin E-cdk2 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. Paradoxically, even though both TCDD and DHT increase p21 expression levels, combined treatment of LNCaP cells with TCDD and DHT results in the reduction of p21 to nearly undetectable levels. Low levels of p21 do not effectively promote cyclin D1-cdk4 complex formation and, although for different causes than p21 overexpression, 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. Alternatively, 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, cell cycle arrest. A similar effect might also result from the reduced levels of cyclin D1 in cells exposed to TCDD+DHT in comparison to DHT alone. Lower levels of cyclin D1 will decrease the levels of functional cyclin D1/cdk4 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-cdk4 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) (see also Fig. 3). TCDD blocks estradiol, TGF-alpha, EGF, IGF-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 that 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 appears 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.

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

ACKNOWLEDGMENTS

We thank Erik Knudsen for the gift of plasmids and James Lessard for the gift of anti-β-actin antibodies. We also thank George Babcock and Sandy Schwemberger for carrying out FACS analyses.

REFERENCES

Abbott BD, Schmid J E, Pitt J A, Buckalew A R, Wood C R, Held G A and Diliberto J J (1999) Adverse Reproductive Outcomes in the Transgenic Ah Receptor-Deficient Mouse. *Toxicol Appl Pharmacol* **155**:62-70.

Astroff B and Safe S (1990) 2,3,7,8-Tetrachlorodibenzo-p-Dioxin As an Antiestrogen: Effect on Rat Uterine Peroxidase Activity. *Biochem Pharmacol* **39**:485-488.

Bakiri L, Lallemand D, Bossy-Wetzel E and Yaniv M (2000) Cell Cycle-Dependent Variations in C-Jun and JunB Phosphorylation: a Role in the Control of Cyclin D1 Expression. *EMBO J* **19**:2056-2068.

Chang C-Y and Puga A (1998) Constitutive Activation of the Aromatic Hydrocarbon Receptor. *Mol Cell Biol* **18**:525-535.

Cheng M, Olivier P, Diehl J A, Fero M, Roussel M F, Roberts J M and Sherr C J (1999) The P21(Cip1) and P27(Kip1) CDK 'Inhibitors' Are Essential Activators of Cyclin D-Dependent Kinases in Murine Fibroblasts. *EMBO J* **18**:1571-1583.

Cleutjens KB, van der Korput H A, Ehren-van Eekelen C C, Sikes R A, Fasciana C, Chung L W and Trapman J (1997) A 6-Kb Promoter Fragment Mimics in Transgenic Mice the Prostate-Specific and Androgen-Regulated Expression of the Endogenous Prostate-Specific Antigen Gene in Humans. *Mol Endocrinol* **11**:1256-1265.

Coqueret O (2003) New Roles for P21 and P27 Cell-Cycle Inhibitors: a Function for Each Cell Compartment? *Trends Cell Biol* **13**:65-70.

Fernandez P and Safe S (1992) Growth Inhibitory and Antimitogenic Activity of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) in T47D Human Breast Cancer Cells. *Toxicology Letters* **61**:185-197.

Ge N-L and Elferink C J (1998) A Direct Interaction Between the Aryl Hydrocarbon Receptor and Retinoblatoma Protein. *J Biol Chem* **273**:22708-22713.

Gottlicher M, Cikryt P and Wiebel F J (1990) Inhibition of Growth by 2,3,7,8-Tetrachlorodibenzo-p-Dioxin in 5L Rat Hepatoma Cells Is Associated With the Presence of Ah Receptor. *Carcinogenesis* **11**:2205-2210.

Griffiths K, Morton M S and Nicholson R I (1997) Androgens, Androgen Receptors, Antiandrogens and the Treatment of Prostate Cancer. *Eur Urol* **32 Suppl 3**:24-40.

Hankinson O (1995) The Aryl Hydrocarbon Receptor Complex. *Annu Rev Pharmacol Toxicol* **35**:307-340.

Hoffer A, Chang C-Y and Puga A (1996) Dioxin Induces *Fos* and *Jun* Gene Expression by Ah Receptor Dependent- and Independent- Pathways. *Toxicol Appl Pharmacol* **141**:238-247.

Jana NR, Sarkar S, Ishizuka M, Yonemoto J, Tohyama C and Sone H (1999) Cross-Talk Between 2,3,7,8-Tetrachlorodibenzo-p-Dioxin and Testosterone Signal Transduction Pathways in LNCaP Prostate Cancer Cells. *Biochem Biophys Res Commun* **256**:462-468.

Johnson L, Dickerson R, Safe S H, Nyberg C L, Lewis R P and Welsh T H J (1992) Reduced Leydig Cell Volume and Function in Adult Rats Exposed to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Without a Significant Effect on Spermatogenesis. *Toxicology* **76**:103-118.

Kharat I and Saatcioglu F (1996) Antiestrogenic Effects of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Are Mediated by Direct Transcriptional Interference With the Liganded Estrogen Receptor. Cross-Talk Between Aryl Hydrocarbon- and Estrogen-Mediated Signaling. *J Biol Chem* **271**:10533-10537.

Klinge CM, Kaur K and Swanson H I (2000) The Aryl Hydrocarbon Receptor Interacts With Estrogen Receptor Alpha and Orphan Receptors COUP-TFI and ERRalpha1. *Arch Biochem Biophys* **373**:163-174.

Knudsen KE, Arden K C and Cavenee W K (1998) Multiple G1 Regulatory Elements Control the Androgen-Dependent Proliferation of Prostatic Carcinoma Cells. *J Biol Chem* **273**:20213-20222.

Knudsen KE, Cavenee W K and Arden K C (1999a) D-Type Cyclins Complex With the Androgen Receptor and Inhibit Its Transcriptional Transactivation Ability. *Cancer Res* **59**:2297-2301.

Knudsen KE, Weber E, Arden K C, Cavenee W K, Feramisco J R and Knudsen E S (1999b) The Retinoblastoma Tumor Suppressor Inhibits Cellular Proliferation Through Two Distinct Mechanisms: Inhibition of Cell Cycle Progression and Induction of Cell Death. *Oncogene* **18**:5239-5245.

Kolluri SK, Weiss C, Koff A and Göttlicher M (1999) $P27^{kip1}$ Induction and Inhibition of Proliferation by the Intracellular Ah Receptor in Developing Thymus and Hepatoma Cells. *Genes Dev* **13**:1742-1753.

Lee HJ and Chang C (2003) Recent Advances in Androgen Receptor Action. *Cell Mol Life Sci* **60**:1613-1622.

Lin TM, Ko K, Moore R W, Simanainen U, Oberley T D and Peterson R E (2002) Effects of Aryl Hydrocarbon Receptor Null Mutation and in Utero and Lactational 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Exposure on Prostate and Seminal Vesicle Development in C57BL/6 Mice. *Toxicol Sci* **68**:479-487.

Lu J and Danielsen M (1998) Differential Regulation of Androgen and Glucocorticoid Receptors by Retinoblastoma Protein. *J Biol Chem* **273**:31528-31533.

Mably TA, Bjerke D L, Moore R W, Gendron-Fitzpatrick A and Peterson R E (1992) In Utero and Lactational Exposure of Male Rats to 2,3,7,8- Tetrachlorodibenzo-p-Dioxin. 3. Effects on Spermatogenesis and Reproductive Capability. *Toxicol Appl Pharmacol* **114**:118-126.

Moore RW, Potter C L, Theobald H M, Robinson J A and Peterson R E (1985) Androgenic Deficiency in Male Rats Treated With 2,3,7,8-Tetrachlorodibenzo-p-Dioxin. *Toxicol Appl Pharmacol* **79**:99-111.

Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanagisawa J, Fujii-Kuriyama Y and Kato S (2003) Modulation of Oestrogen Receptor Signalling by Association With the Activated Dioxin Receptor. *Nature* **423**:545-550.

Porterfield SP (1994) Vulnerability of the Developing Brain to Thyroid Abnormalities: Environmental Insults to the Thyroid System. [Review]. *Environ Health Perspect* **102 Suppl 2**:125-130.

Puga A, Barnes S J, Dalton T P, Chang C, Knudsen E S and Maier M A (2000) Aromatic Hydrocarbon Receptor Interaction With the Retinoblastoma Protein Potentiates Repression of E2F-Dependent Transcription and Cell Cycle Arrest. *J Biol Chem* **275**:2943-2950.

Puga A, Nebert D W and Carrier F (1992) Dioxin Induces Expression of C-*Fos* and C-*Jun* Proto-Oncogenes and a Large Increase in Transcription Factor AP-1. *DNA Cell Biol* **11**:269-281.

Puga A, Xia Y and Elferink C (2002) Role of the Aryl Hydrocarbon Receptor in Cell Cycle Regulation. *Chem Biol Interact* **141**:117-130.

Roman BL, Pollenz R S and Peterson R E (1998) Responsiveness of the Adult Male Rat Reproductive Tract to 2,3,7,8- Tetrachlorodibenzo-p-Dioxin Exposure: Ah Receptor and ARNT Expression, CYP1A1 Induction, and Ah Receptor Down-Regulation. *Toxicol Appl Pharmacol* **150**:228-239.

Roman BL, Sommer R J, Shinomiya K and Peterson R E (1995) In Utero and Lactational Exposure of the Male Rat to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin: Impaired Prostate Growth and Development Without Inhibited Androgen Production. *Toxicol Appl Pharmacol* **134**:241-250.

Saatcioglu F, Perry D J, Pasco S and Fagan J B (1990) Multiple DNA-Binding Factors Interact With Overlapping Specificities at the Aryl Hydrocarbon Response Element of the Cytochrome P450IA1 Gene. *Mol Cell Biol* **10**:6408-6416.

Sherr CJ and Roberts J M (1999) CDK Inhibitors: Positive and Negative Regulators of G1-Phase Progression. *Genes Dev* **13**:1501-1512.

Strobeck MW, Fribourg A F, Puga A and Knudsen E S (2000) Restoration of Retinoblastoma Mediated Signaling to Cdk2 Results in Cell Cycle Arrest. *Oncogene* **19**:1857-1867.

Theobald HM and Peterson R E (1997) In Utero and Lactational Exposure to 2,3,7,8-Tetrachlorodibenzo-Rho-Dioxin: Effects on Development of the Male and Female Reproductive System of the Mouse. *Toxicol Appl Pharmacol* **145**:124-135.

Wang F, Samudio I and Safe S (2001) Transcriptional Activation of Cathepsin D Gene Expression by 17beta-Estradiol: Mechanism of Aryl Hydrocarbon Receptor-Mediated Inhibition. *Mol Cell Endocrinol* **172**:91-103.

Wang W, Smith I R and Safe S (1998) Aryl Hydrocarbon Receptor-Mediated Antiestrogenicity in MCF-7 Cells: Modulation of Hormone-Induced Cell Cycle Enzymes. *Arch Biochem Biophys* **356**:239-248.

Weiss C, Kolluri S K, Kiefer F and Gottlicher M (1996) Complementation of Ah Receptor Deficiency in Hepatoma Cells: Negative Feedback Regulation and Cell Cycle Control by the Ah Receptor. *Exp Cell Res* **226**:154-163.

Yeh S, Miyamoto H, Nishimura K, Kang H, Ludlow J, Hsiao P, Wang C, Su C and Chang C (1998) Retinoblastoma, a Tumor Suppressor, Is a Coactivator for the Androgen Receptor in Human Prostate Cancer DU145 Cells. *Biochem Biophys Res Commun* **248**:361-367.

Zacharewski TR, Bondy K L, McDonell P and Wu Z F (1994) Antiestrogenic Effect of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin on 17 Beta-Estradiol-Induced PS2 Expression. *Cancer Res* **54**:2707-2713.

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES

*This work was supported by NIH Grants ES06273 and ES10807 (A.P.), CA09996 and CA93404 (K.E.K). and P30 ES06096 (A.P. and K.E.K.). S.B.-E. was supported by a predoctoral NRSA grant from the National Institute of Environmental Health Sciences.

Address reprint requests to: Alvaro Puga, Department of Environmental Health. University of Cincinnati Medical Center. P.O. Box 670056. Cincinnati, OH 45367-00567. Phone: (513) 558-0916; FAX: (513) 558-0925.

E-mail: Alvaro.Puga@UC.EDU

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS TO FIGURES

Fig. 1. Schematic representation of *PSA* and p21$^{CIP1/WAF1}$ regulatory sequences and luciferase reporters derived therefrom. The *top* diagram illustrates the upstream regulatory domain of the human *PSA* gene from coordinates -6000 to the transcriptional start site at +1. *Closed* and *hatched boxes* denote AHRE and ARE motifs, respectively. The *bottom* diagram, 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 fist exon is represented by the *stippled box* between coordinates +1 and +90. *Closed* boxes represent AHRE motifs and *hatched boxes* p53 responsive sites.

Fig 2. Inhibition of androgen-dependent cell proliferation in LNCaP cells by TCDD. (A) Cells grown in Improved Minimum Essential Medium (IMEM) supplemented with 10% complete serum were seeded at 1X10^5 cells/well in poly-L-lysine coated 6-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 X 10^6 cells/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 Pharmacia Biotech) for 5-6 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 (FACS). (C) 2 X 10^4 cells/well were seeded in poly-l-lysine coated 6 well plates in 5% steroid depleted fetal bovine serum (CDT) and allowed to attach for 24 h prior to 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).

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

Experiments were done at least in duplicate with 100 events scored per sample. Means ± SD are represented for each experiment. The *asterisk* indicates significant differences from control at $p<0.05$.

Fig 3. DHT-induced cyclin D1 expression and RB phosporylation are decreased in the presence of TCDD. LNCaP cells were propagated in 5% CDT for 72 h prior to 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 employing specific polyclonal antibodies were used in detecting protein levels of cyclin D1, hypophosphorylated RB, and $P21^{WAF1/CIP1}$.

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 construct, 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 plasmids expressing AHR and ARNT. Cells were treated with 10 nM TCDD for 24 hr and luciferase activity was determined. To characterize the role of p53 in TCDD-induced p21 expression, cells were co-transfected with pCMVp53 (Fig. 4B) followed by treatment with 10 nM TCDD post-transfection for 36 h. Luciferase and β-galactosidase activities were measured as described in *Material and Methods*. Luciferase values were normalized to β-galactosidase activities and are represented as Mean ± SD.

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

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

medium for three days prior to 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 employed and visualized with 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.

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 co-transfected 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. Following transfection, cells were treated with 0.1% vehicle or with the indicated androgen in the absence (stripped bars) or presence (solid bars) of 10 nM TCDD for 48 h. Luciferase and β-galactosidase activities were measured as described in *Material and Methods*. Luciferase values were normalized to β-galactosidase activities and are presented as the mean ± SD.

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 each of the reporter

Molecular Pharmacology Fast Forward. Published on May 26, 2004 as DOI: 10.1124/mol.104.000356
This article has not been copyedited and formatted. The final version may differ from this version.

constructs indicated in each panel 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 ± SD.

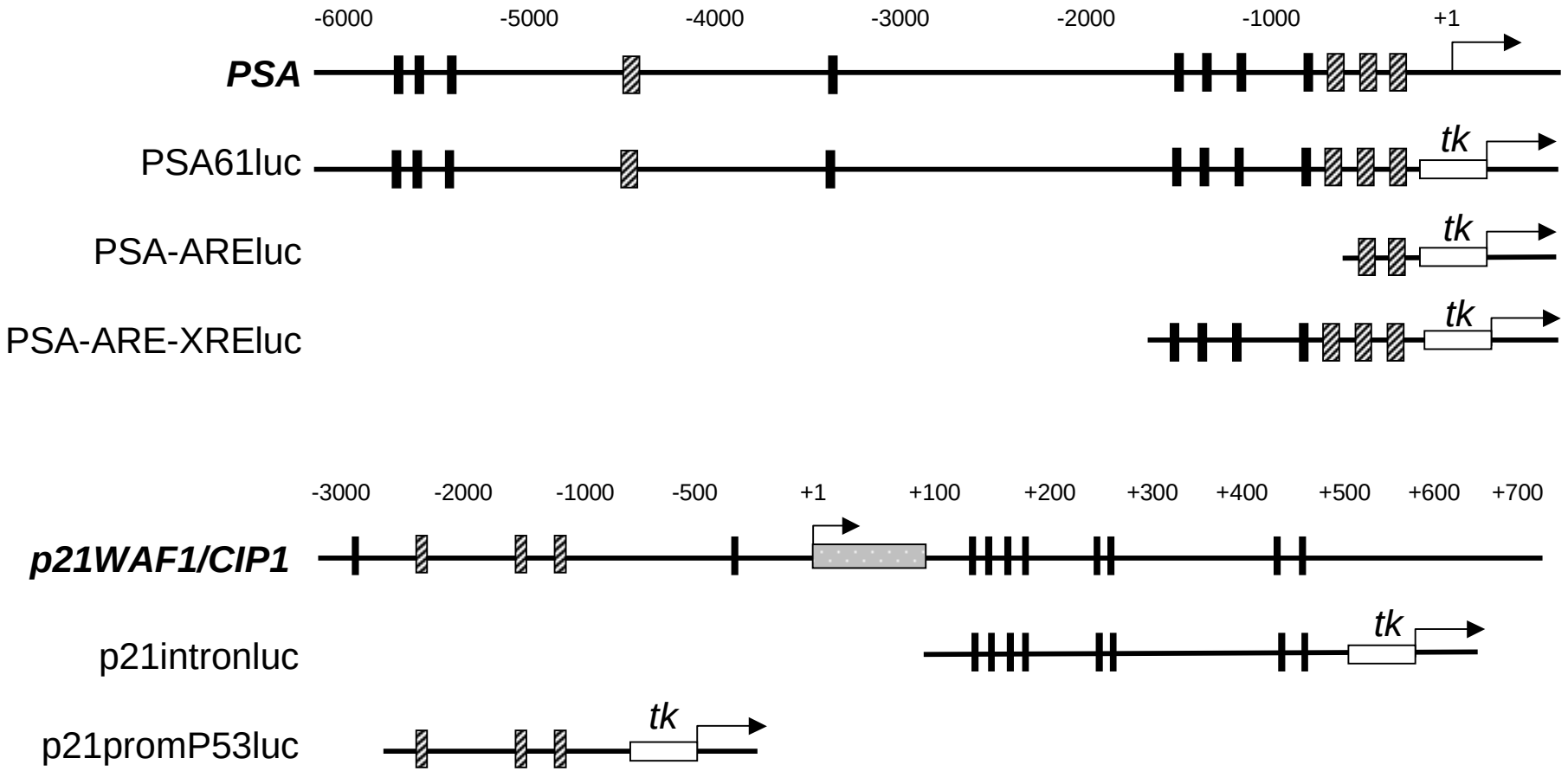

Fig. 1. Barnes-Ellerbe

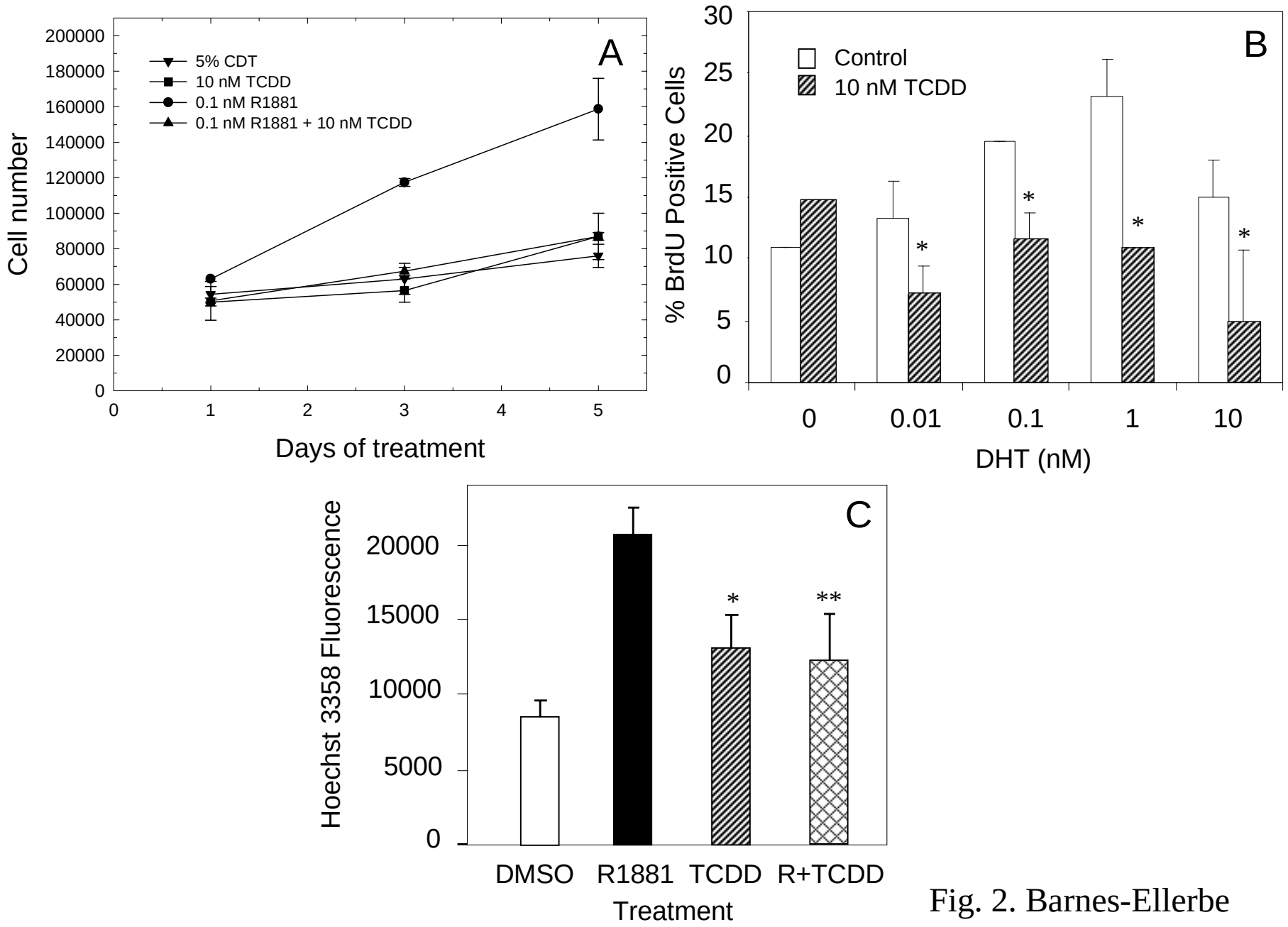

Fig. 2. Barnes-Ellerbe

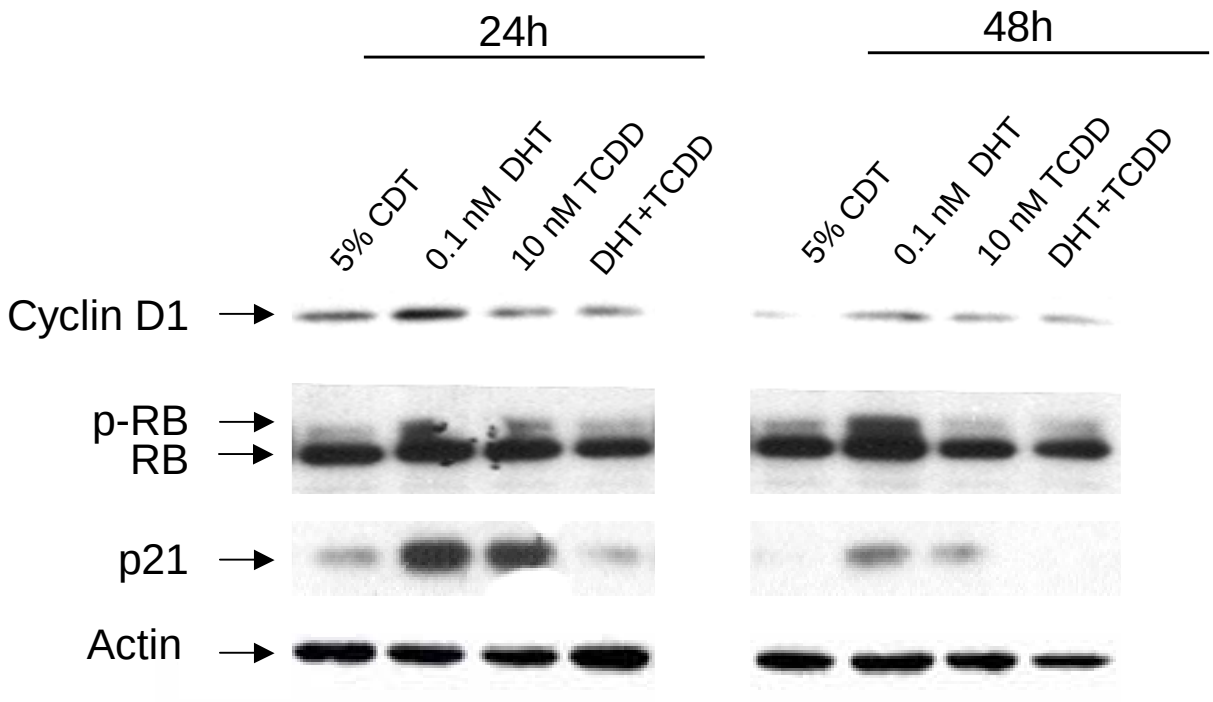

Fig. 3. Barnes-Ellerbe

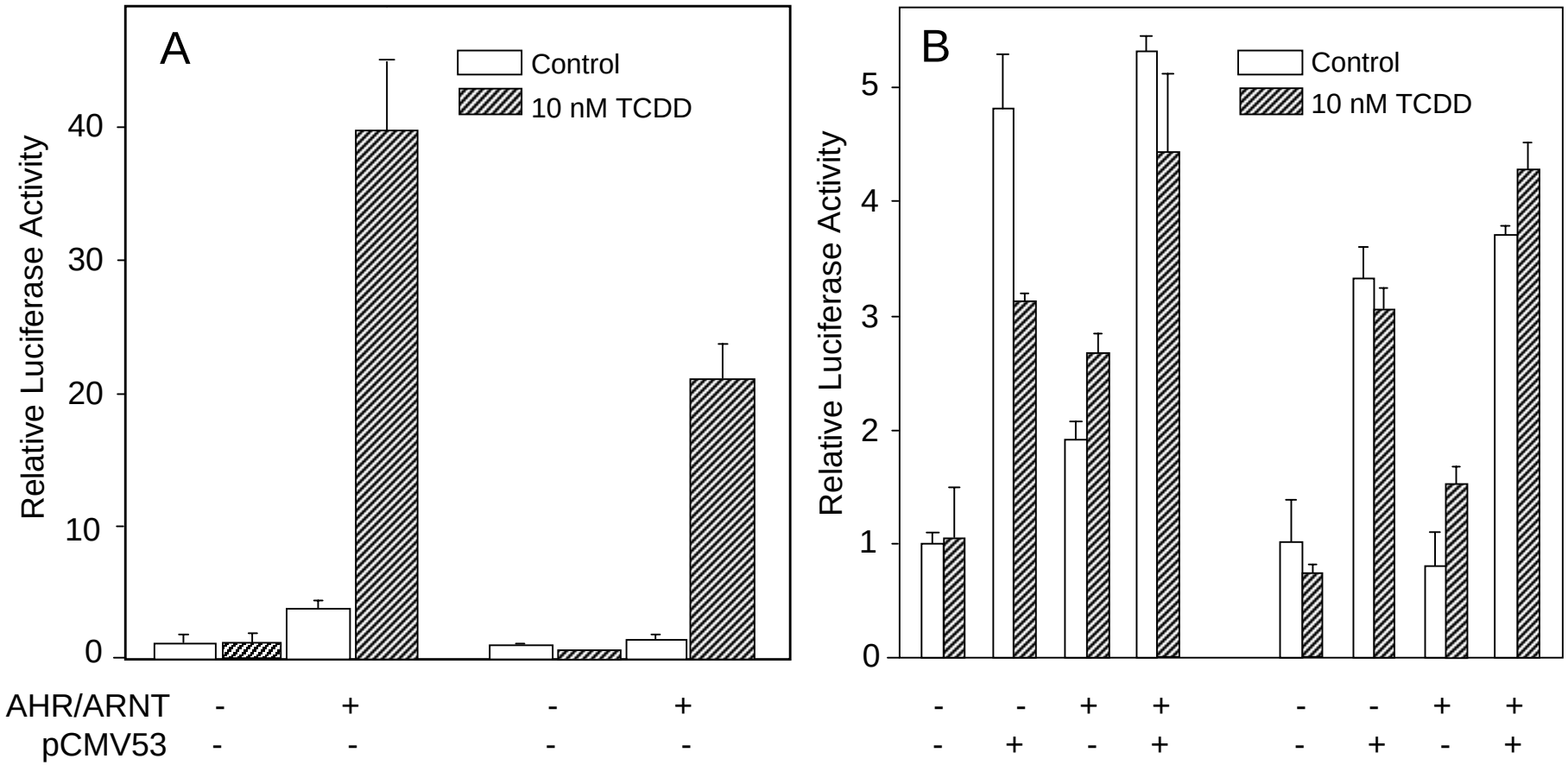

Fig 4. Barnes-Ellerbe

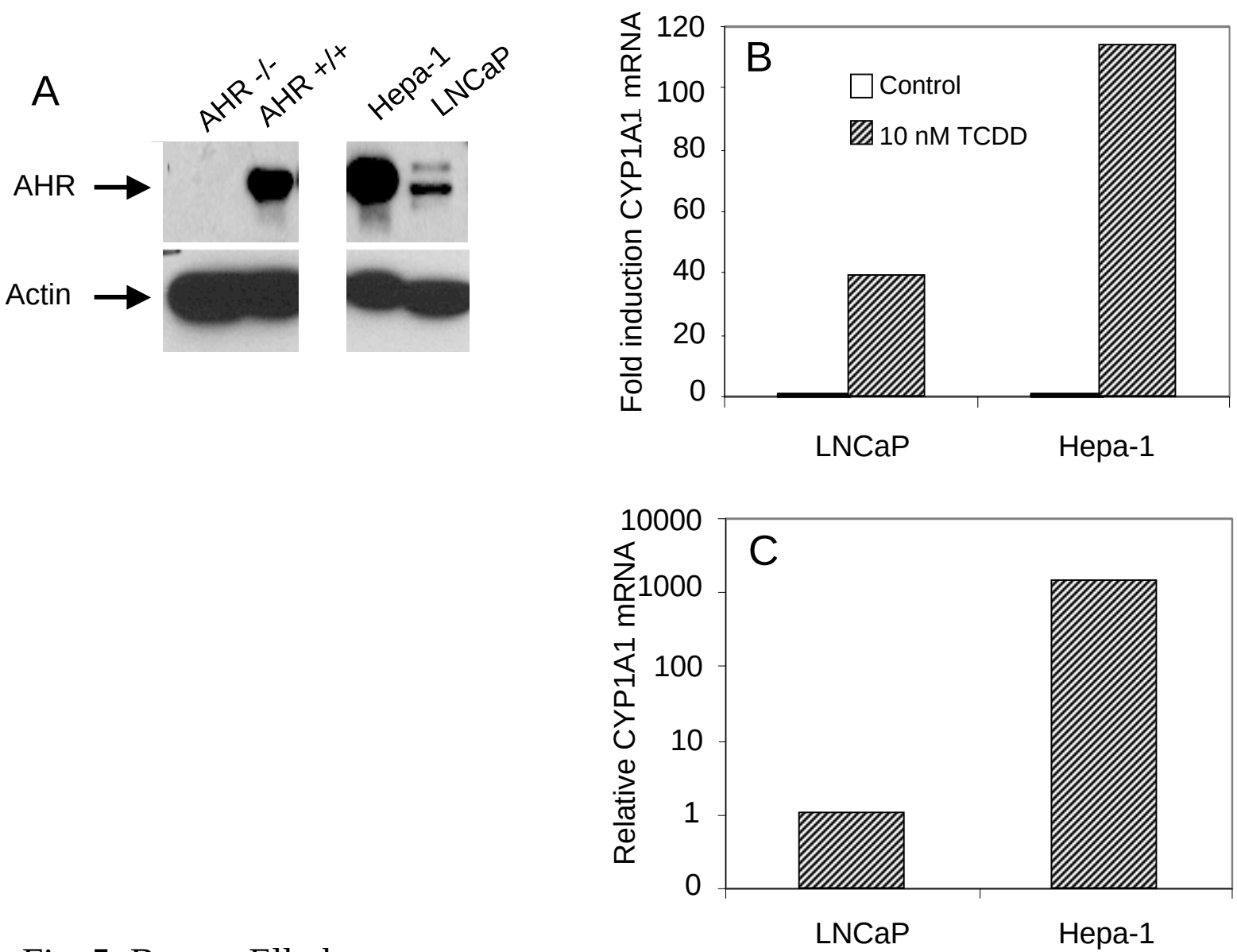

Fig. 5 Barnes-Ellerbe

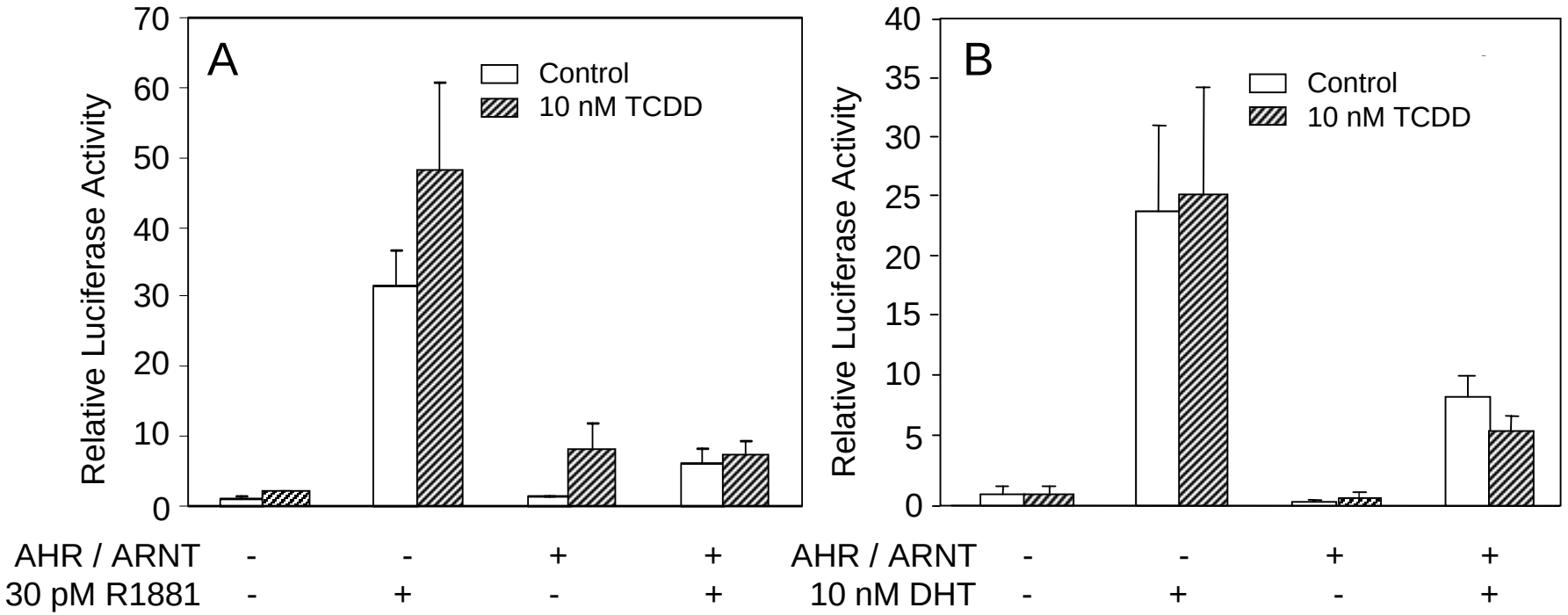

Fig. 6. Barnes-Ellerbe

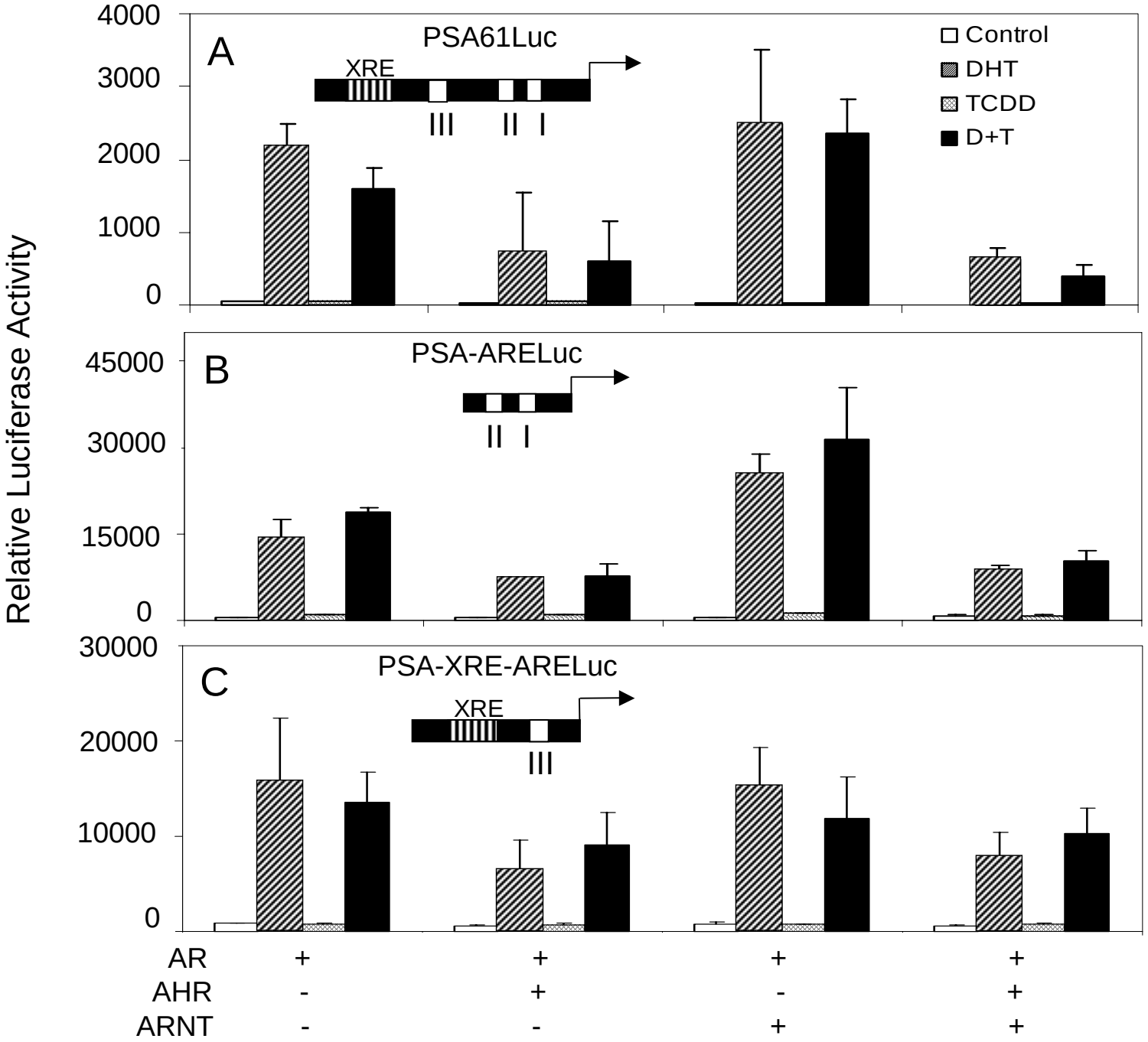

Fig. 7.
Barnes-Ellerbe