Full Paper

Repression of Activated Aryl Hydrocarbon Receptor–Induced Transcriptional Activation by 5 α -Dihydrotestosterone in Human Prostate Cancer LNCaP and Human Breast Cancer T47D CellsNoriko Sanada¹, Yuka Gotoh¹, Rumiko Shimazawa^{1,#}, Carolyn M. Klinge², and Ryoichi Kizu^{1,*}¹Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts, Kodo, Kyotanabe, Kyoto 610-0395, Japan²Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, KY 40292, USA

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Abstract. Polycyclic aromatic hydrocarbons (PAHs) and dioxins are ubiquitous environmental pollutants and activate the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor. It has been reported that testosterone represses 2,3,7,8-tetrachlorodibenzo-*p*-dioxin–induced transcription of the cytochrome P450 (CYP) 1A1 gene in LNCaP cells. In this study, we investigated the mechanism for the repression of 3-methylcholanthrene (3MC)-induced transcription of AhR-regulated genes, CYP1A1, CYP1A2, CYP1B1, and AhR repressor (AhRR), by 5 α -dihydrotestosterone (DHT) in LNCaP and T47D cells, which are androgen receptor (AR)- and AhR-positive. Real-time PCR analysis showed that DHT repressed 3MC-induced mRNA expression of the CYP1 family and AhRR genes. DHT repressed 3MC-induced luciferase activity in an AhR response element–driven luciferase reporter assay in LNCaP and T47D cells. The inhibitory effect of DHT was abolished by knockdown of AR protein with siRNA. The protein levels of AhR and AhR nuclear translocator (Arnt), the AhR-dimerizing partner, were not affected by DHT. Co-immunoprecipitation assay showed that DHT significantly facilitated the complex formation between AR and AhR in 3MC-treated cells. These results suggest that complex formation between activated AR and AhR plays an important role in the suppression of 3MC-induced transcription of CYP1 family genes by DHT.

Keywords: androgen receptor, aryl hydrocarbon receptor, cross-talk, complex formation, transcriptional activation

Introduction

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that binds to various environmental pollutants containing polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons such as dioxins (1). These compounds are found ubiquitously in the environment and are potentially hazardous to human and animal health. Most of the toxic effects like endocrine-disrupting action and carcinogenesis are mediated by AhR (2–7). Furthermore, AhR regulates normal

development of tissues, for example, reproductive tissues and liver, and diverse biological processes, including lipid synthesis, adipocyte differentiation, cell cycle progression, and immune system function (5, 8–11).

AhR is a member of the basic helix-loop-helix / Per-Arnt-Sim (bHLH/PAS) family of transcription factors (12). Other members of this family include Period (Per), AhR nuclear translocator (Arnt), Single-minded gene product (Sim), Clock, hypoxia-inducible factor 1 α (HIF1 α), and brain and muscle Arnt-like protein (BMAL). Unliganded AhR exists in a complex with heat shock protein 90 (HSP90), the HSP90 co-chaperone p23, and a hepatitis B virus X-associated protein 2 (XAP2) in the cytoplasm (13–15). Upon ligand binding, activated AhR is translocated from the cytoplasm into the nucleus and dimerizes with Arnt. The AhR/Arnt heterodimer binds to a specific DNA element,

[#]Present address: Innovation of New Biomedical Engineering Center, Tohoku University, Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

*Corresponding author. rkizu@dwc.doshisha.ac.jp

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termed the xenobiotic response element (XRE), in the transcriptional regulatory region to regulate the transcription of AhR target genes including cytochrome P450 (CYP) 1A1, CYP1A2, CYP1B1, and AhR repressor genes (16–18). These oxidative enzymes, CYP1A1, CYP1A2, and CYP1B1, metabolize PAHs to ultimate carcinogenic species.

Androgens regulate various functions during normal development and in adulthood. Especially, androgen is necessary for the development and maintenance of male reproductive organs including prostate, seminal vesicle, epididymis, and testis. Most of androgen's actions are mediated by the androgen receptor (AR). AR is a ligand-activated transcription factor and belongs to the steroid/thyroid hormone nuclear receptor superfamily (19). Unliganded AR predominantly exists in the cytoplasm. Upon ligand binding, activated AR is translocated into the nucleus where it recognizes and binds to a specific DNA element, termed the androgen response element (ARE), to regulate the transcription of AR target genes.

Dioxins and PAHs, acting as AhR ligands, are well-known disruptors of endocrine functions. There are numerous reports that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]pyrene (BaP), and 3-methylcholanthrene (3MC) repress AR-responsive biological processes. *In utero* and lactational exposures of male rats to TCDD decreased the weight of prostate, seminal vesicle, and epididymus (8, 20, 21). *In utero* and lactational exposure of male rats to TCDD inhibits prostatic epithelial differentiation, formation of the epithelial bud to develop into secretory glands, and androgen-responsive mRNA expression in the prostate (22, 23). In human prostate cancer cells, TCDD, BaP, and 3MC repress androgen-induced gene transcription and cell proliferation (24–29). Importantly, TCDD exerts inhibitory effects on prostate growth and development without inhibiting testicular androgen production (21, 23). Furthermore, we have reported that PAHs do not bind to AR (26). Cross-talk between AR- and AhR-mediated signal transduction pathways plays an important role in the inhibitory effects of TCDD. Recent studies have shown several ways of cross-talk between AR- and AhR-mediated signal transduction pathways: 1) inhibition of androgen-induced transcriptional activation by AhR agonists, 2) transcriptional activation of androgen-regulated genes by AhR agonists in the absence of androgens, and 3) inhibition of AhR agonist-induced transcriptional activity by androgens.

First, an AhR agonist inhibited androgen-induced transcriptional activation by a mechanism involving complex formation between activated AhR and AR in which AhR acts as an E3 ubiquitin ligase to degrade AR protein (29). Second, in LNCaP cells, PAH caused

induction of ARE-driven luciferase activity in the absence of androgens. For the mechanisms of the stimulatory effect of AhR agonists on androgen-regulated gene, it is proposed that unliganded AR forms a complex with activated AhR and binds to ARE in the transcriptional regulatory region of AR-regulated genes (30). Third, TCDD-induced CYP1A1 mRNA expression and CYP1A1 enzymatic activity, which was measured as ethoxyresorufin *O*-deethylase (EROD) activity, were inhibited by testosterone in LNCaP cells (24, 25). Also, TCDD-induced AhR-responsive luciferase reporter activity was inhibited by DHT in COS monkey kidney cells (31). However, the mechanism for the inhibitory effect of androgens on PAH-induced transcription of AhR-regulated genes has not yet been confirmed. Because of the importance of the cross-talk between AR and AhR-mediated signal transduction pathways in the toxic effects of AhR agonists, elucidation of the mechanism by which androgens repress PAH-induced transcription may help clarify AhR and AR functions. In this study, we reported that DHT represses 3MC-induced transcription of CYP1A1, CYP1A2, CYP1B1, and AhRR genes in LNCaP and T47D cells, and our results suggest that complex formation between AR and AhR is important for repression of 3MC-induced gene transcription by DHT.

Materials and Methods

Materials

5 α -Dihydrotestosterone (DHT) of biochemical study grade, 3MC (>99% pure), and dimethyl sulfoxide (DMSO) of ultra pure grade were purchased from Wako Pure Chemicals (Osaka). All other chemicals were of reagent grade or better and were obtained from commercial sources.

Plasmids

pGL3-XRE-SV40, an XRE-driven firefly luciferase-expressing plasmid, was constructed by inserting three tandem repeats of the consensus XRE-containing sequence (5'-TCTTCTCACGCAACTCCGA-3') into the upstream region of the firefly luciferase gene in the pGL3-promoter vector (Promega, Madison, WI, USA). The underlined nucleotides are the consensus XRE. The sequence of pGL3-XRE-SV40 was confirmed by restriction enzymes digestion and by DNA sequencing (ABI PRISM 3130/3130xl; Applied Biosystems, Foster City, CA, USA). *Renilla* luciferase expression pRL-SV40 plasmid was purchased from Promega.

Cell culture and treatment

Human prostate cancer cell line LNCaP-FGC and

human breast cancer cell line T47D were purchased from American Type Culture Collection (Manassas, VA, USA). LNCaP and T47D cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France), 100 units/ml penicillin, and 100 µg/ml streptomycin and were passaged with trypsinization every four days. Cell monolayers were maintained in an atmosphere of 5% CO₂ and 95% air under a saturated humidity at 37°C. In assays, the cells were plated in 10-cm diameter dishes at a cell density of 1.8×10^6 cells/dish and cultured for 48 h in the growth medium. The cells were washed with phosphate-buffered saline (PBS) and cultured for 24 h in an assay medium [phenol red-free DMEM (Sigma) containing 5% charcoal/dextran-treated FBS (Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin]. The cells were then treated with 0.1% DMSO, 1 µM 3MC alone (3MC treatment), or in a combination with 10 nM DHT (3MC/DHT treatment). Stock solutions of 3MC and DHT were prepared using DMSO and the final DMSO concentration in medium did not exceed 0.1% (v/v).

Reverse transcription and real-time PCR

After the treatment, the cells were washed twice with PBS. Total RNA was extracted from the cells by using the RNeasy mini kit (Qiagen, Chatsworth, CA, USA). Reverse transcription was performed by using the ExScript RT reagent kit (Takara, Shiga) according to the manufacturer's instructions. Quantitative real-time PCR was carried out using SYBR premix ExTaq (Takara) on a Smart Cycler II System (Cepheid, Sunnyvale, CA, USA). Primer sequences are listed in Table 1. PCR conditions for repeated cycles were as follows: for CYP1A1 and CYP1A2, 95°C for 15 s and 60°C for 1 min; for CYP1B1, 95°C for 15 s and 65°C for 30 s; for AhRR, 95°C for 15 s, 56°C for 5 s, and 72°C for 8 s; for

RPLP0, 95°C for 5 s and 66°C for 20 s. The RPLP0 product was used as a control to assure that equal amounts of RNA were used.

Dual luciferase reporter assay

LNCaP cells were plated at a cell density of 1.2×10^5 cells/well in 12-well plates and cultured for 48 h in growth medium. The cells were then washed with PBS and transiently transfected with pGL3-XRE-SV40 (0.8 µg/well) and pRL-SV40 (0.04 µg/well) using the lipofection reagent Lipofectamine (Invitrogen, Carlsbad, CA, USA) for 5 h at 37°C, according to the manufacturer's instructions. The cells were washed once with PBS and cultured in the fresh assay medium. At 48-h post-transfection, cells were treated 12 h with DHT and 3MC at the given concentrations. Luciferase activity was determined with the PicaGene Dual Sea Pansy Luminescence Kit (Toyo Ink, Tokyo). Firefly luciferase activity was normalized to the *Renilla* luciferase activity.

Whole cell and nuclear extracts preparation

After the treatment with 3MC or 3MC/DHT, whole cell and nuclear extracts were prepared using CellLytic M and CellLytic NuCLEAR Extraction Kits (Sigma), respectively, according to the manufacturer's instructions. Protein concentrations were determined using Protein Assay CBB Solution (5×) (Nacalai Tesque, Kyoto).

siRNA treatment

AR siRNA (5'-gaccuaccgaggagcuuucdTdT-3') was directed against nucleotides 51-71 downstream of the start codon of the human AR gene (32). A negative control (non-silencing) siRNA (5'-uuccuccgaacgugucagudTdT-3'), purchased from Qiagen, was used as a negative control. Cells were plated in 10-cm diameter dishes at a cell density of 1.2×10^6 cells/dish and cultured for 48 h in growth medium. The cells were then washed with PBS and transfected with siRNA for non-silencing or AR at an siRNA concentration of 100 nM for 24 h using RNAiFect Transfection Reagent (Qiagen) in Opti-MEM serum-free medium (Invitrogen), according to the manufacturer's instructions. Cells were then subjected to the quantitative real-time PCR assay.

Western blotting

Whole cell and nuclear extracts containing equal amounts of protein were separated by SDS-PAGE (5% – 20% acrylamide gel) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membrane was blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat milk and then probed with rabbit anti-human AhR polyclonal

Table 1. Primers used in this study

Gene		Primer sequence (5'-3')
CYP1A1	Forward	GTCATCTGTGCCATTTGCTTTG
	Reverse	CAACCACCTCCCCGAAATTATT
CYP1A2	Forward	TGTTCAAGCACAGCAAGAAGG
	Reverse	TGCTCCAAAGACGTCATTGAC
CYP1B1	Forward	AACGTCATGAGTGCCGTGTGT
	Reverse	GGCCGGTACGTTCTCCAAATC
AhRR	Forward	ACCGCGGATGCAAAAGTAAAAG
	Reverse	GCTCCTTCCTGCTGAGTAATTGG
RPLP0	Forward	GCGACCTGGAAGTCCAAC
	Reverse	CCATCAGCACCACAGCCTTC

antibody (sc-5579; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human AR polyclonal antibody (06-680, Millipore), rabbit anti-human Arnt polyclonal antibody (sc-5580, Santa Cruz Biotechnology), or rabbit anti-human RPLP0 polyclonal antibody (HPA003512, Sigma), followed by peroxidase-conjugated secondary anti-mouse or rabbit immunoglobulin (IgG) antibodies (GE Healthcare UK, Ltd., Buckinghamshire, England). Membranes were then visualized with chemiluminescent detection reagents: ECL Plus (GE Healthcare) for AhR and Chemi-Lumi One (Nacalai Tesque) for AR, Arnt, and RPLP0, by using LumiVision PRO 400EX (Aisin Seiki, Aichi). LumiVision Analyzer 400 (Aisin Seiki) was used to digitalize and analyze the relative amounts of protein, based on pixel density, in the immunoblot bands.

Co-immunoprecipitation

Nuclear extracts were initially precleared by incubation with Protein G Sepharose 4 Fast Flow, protein G-coupled Sepharose beads (GE Healthcare). The clarified nuclear extracts were then incubated with goat anti-human AhR polyclonal antibody (sc-8088, Santa Cruz Biotechnology) in immunoprecipitation buffer [20 mM Hepes-NaOH (pH 7.5), 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, 60 mM β -glycerophosphate, 20 mM NaF, 0.5% Tween 20, 0.5% IGEPAL CA-630 (Sigma), 0.5% sodium deoxycholate, 1 mM DTT, protease inhibitor cocktail (Sigma)] under gentle agitation overnight at 4°C. Protein G Sepharose 4 Fast Flow beads were then added to the immunocomplexes and incubated for 2 h under gentle agitation at 4°C. AR and AhR proteins in the precipitates were analyzed by the western blotting technique described above.

Statistical analyses

Statistical analyses were performed by the unpaired Student's *t*-test, whereby a value of $P < 0.05$ was considered to be significant.

Results

Repression of 3MC-induced transcription by DHT in AR- and AhR-positive cells

It has reported that the inhibitory effect of testosterone was evaluated on the CYP1A1 gene in LNCaP cells (24). It is uncertain whether testosterone represses transcription of AhR-regulated genes other than CYP1A1. First of all, we examined the effect of DHT on PAH-induced transcription of AhR-regulated genes, CYP1A2, CYP1B1, and AhRR in addition to CYP1A1, in LNCaP and T47D cells by the quantitative real-time PCR technique. The results for LNCaP cells are shown

Fig. 1. Repression of 3MC-induced mRNA expression of CYP1A1, CYP1A2, CYP1B1, and AhRR genes by DHT in LNCaP (A) and T47D (B) cells. Cells were treated with 0.1% DMSO, 1 μ M 3MC, or 1 μ M 3MC in combination with 10 nM DHT for 12 h. CYP1A1, CYP1A2, CYP1B1, and AhRR mRNA levels were measured by real-time PCR. The RPLP0 product was analyzed as a control to assure that equal amounts of RNA were used. Each column and vertical bar represents the mean $\pm$ S.D. of three separate experiments. * and **: Significantly different at $P < 0.05$ and $P < 0.01$, respectively. N.D. = not detected.

in Fig. 1A. 3MC increased the mRNA levels of CYP1A1 and CYP1A2, and their inductions were significantly repressed by DHT by approximately 70% and 90%, respectively. CYP1B1 mRNA level was not induced by 3MC as noted in previous reports (25). AhRR mRNA was not detected in LNCaP cells. In T47D cells, 3MC increased the mRNA levels of all four genes, and the inductions of CYP1A1, CYP1A2, CYP1B1, and AhRR mRNA levels were repressed by DHT by approximately 25%, 75%, 80%, and 75%, respectively.

We performed dual luciferase reporter assays using pGL3-XRE-SV40, an XRE-driven luciferase expression vector. 3MC-induced XRE-driven luciferase activity in LNCaP and T47D cells (Fig. 2). DHT repressed this induction of luciferase activity in LNCaP and T47D cells by approximately 90% and 50%, respectively.

Involvement of AR in the inhibitory effect of DHT

To determine whether AR is required for the inhibitory

Fig. 2. Repression of 3MC-induced XRE-driven luciferase activity by DHT in LNCaP (A) and T47D (B) cells. LNCaP and T47D cells were transfected with pGL3-XRE-SV40 XRE-driven firefly luciferase expression vector and pRL-SV40 renilla luciferase expression vector. At 48 h post-transfection, cells were treated with 0.1% DMSO, 1 μ M 3MC alone, or 1 μ M 3MC in combination with 10 nM DHT for 12 h. The firefly luciferase activity was normalized by the Renilla luciferase activity. Each column and vertical bar represents the mean $\pm$ S.D. of four separate experiments. *: Significantly different at $P < 0.05$.

effect of DHT, AR protein was knocked down using siRNA in LNCaP cells. The specificity of AR knockdown was confirmed by western blotting. AR protein levels decreased significantly with time and reached a minimum 4-day post-transfection in AR siRNA-transfected cells, whereas non-silencing siRNA-transfected cells showed no observable change in AR protein levels (Fig. 3A). AR-knockdown cells were subjected to real-time PCR assay using CYP1A1-specific primers. As shown in Fig. 3B, 3MC increased the mRNA level of CYP1A1 in LNCaP cells treated with AR siRNA or non-silencing siRNA. The induction of CYP1A1 mRNA expression was significantly reduced by DHT in non-silencing siRNA-treated LNCaP cells, whereas DHT showed almost no effect on the CYP1A1 mRNA level in the AR siRNA-treated cells. These results indicate that AR protein is necessary for the inhibitory effect of DHT on 3MC-induced transcription of CYP1 family genes.

Effect of DHT on cellular protein levels of AhR and Arnt

To determine if DHT affects the cellular AhR and Arnt protein levels, western blotting of whole cell extracts from 3MC- or 3MC/DHT-treated LNCaP cells was performed. As shown in Fig. 4, while AhR protein levels in whole cell extracts were significantly reduced by 3MC, the AhR protein levels in whole cell extracts were similar in 3MC- and 3MC/DHT-treated cells. Activation of AhR by 3MC brings about reduction in AhR protein level through activation of the ubiquitin-proteasome system (29, 33). Reduction in cellular AhR protein level by 3MC was observed and this result is consistent with reported knowledge. Arnt, the dimeriza-

Fig. 3. Involvement of AR in the inhibitory effect of DHT. LNCaP cells were transfected with the non-silencing siRNA or AR siRNA and then treated with 0.1% DMSO, 1 μ M 3MC alone, or 1 μ M 3MC in combination with 10 nM DHT for 12 h, followed by Western blotting analysis (A) or real-time PCR assay (B). In the Western blotting analysis, AR and RPLP0 protein levels in whole cell extracts were measured. In the real-time PCR assay, CYP1A1 mRNA expression levels were measured. The RPLP0 product was analyzed as a control to assure that equal amounts of RNA were used. Each column and vertical bar represents the mean $\pm$ S.D. of three separate experiments. **: Significantly different at $P < 0.01$.

Fig. 4. Effect of DHT on protein levels of AhR and Arnt. LNCaP cells were incubated with 0.1% DMSO, 1 μ M 3MC alone, or 1 μ M 3MC in combination with 10 nM DHT for 60 min. Whole cell extracts were then prepared and subjected to western blot analyses of AhR and AR. The RPLP0 protein level was analyzed as a control to assure that equal amounts of whole proteins were used.

tion partner of AhR, showed no change at the protein level in whole cell extracts with 3MC- or 3MC/DHT-treatment. These results demonstrate that DHT does not cause a decrease in the cellular protein levels of either AhR or Arnt, which leads to the inhibitory effect of DHT.

Complex formation of AR with AhR

Next, we investigated whether DHT affects the translocation of AhR from the cytoplasm to the nucleus. Figure 5A shows the time-course of AhR levels in nuclear extracts of LNCaP cells following 3MC- or

Fig. 5. Complex formation of AR with AhR in nuclear extracts. **A:** LNCaP cells were treated with 1 μ M 3MC alone or 1 μ M 3MC in combination with 10 nM DHT for the indicated times. Nuclear extracts were prepared and analyzed for AhR by the western blot technique. **B:** LNCaP cells were treated with 0.1% DMSO, 1 μ M 3MC alone, or 1 μ M 3MC in combination with 10 nM DHT for 60 min. Nuclear extracts were prepared and input levels of AR and AhR were examined by Western blot analysis. Nuclear extracts were subjected to the co-immunoprecipitation assay coupled with anti-AhR antibodies. AR and AhR in the immunoprecipitates were analyzed by the Western blot technique. **C:** The AR protein level was normalized to the AhR protein level (AR/AhR). Ratios of AR/AhR protein levels of input control and immunoprecipitates of AhR are shown. Each column and vertical bar represents the mean $\pm$ S.D. of three separate experiments. *: Significantly different at $P < 0.05$.

3MC/DHT-treatment. AhR levels in the nuclear extracts peaked at 40 to 60 min after treatment and then declined slowly. A significant difference in the time-course of nuclear AhR level was not observed between 3MC- and 3MC/DHT treatments.

To determine whether AhR forms a complex with AR in the nucleus of LNCaP cells treated with 3MC or 3MC/DHT, nuclear extracts were immunoprecipitated with an anti-AhR antibody and blotted for AR and AhR. First, we measured the protein levels of AhR and AR in nuclear extracts from 3MC- or 3MC/DHT-treated cells (input control). As shown in Fig. 5B (left panel), 3MC-

treatment increased nuclear AhR and AR protein levels and 3MC/DHT-treatment further increased nuclear AR-protein levels. The nuclear AhR-protein levels were similar in 3MC- and 3MC/DHT-treated cells. AR was co-immunoprecipitated with AhR in nuclear extracts from cells treated with 3MC and 3MC/DHT (Fig. 5B: right panel). While the ratio of AR/AhR was increased with both input control and immunoprecipitates of AhR by co-treatment with DHT, the magnitude of the increase was significantly greater with immunoprecipitates (Fig. 5C). AR was not detected in the immunoprecipitates of the cells treated with 0.1% DMSO. Taken together, these results suggest that DHT significantly facilitated the formation of a complex between AR and AhR in the nucleus of 3MC-treated cells.

Discussion

Several types of cross-talk between AR- and AhR-mediated signal transduction pathways have been reported. The elucidation of the mechanisms for AR-AhR cross-talk may contribute to a better understanding of the function of AR and AhR in normal development and the toxicities of AhR agonists.

One example of AR-AhR cross-talk is the report that testosterone repressed TCDD-induced transcription of AhR-regulated CYP1A1 gene and CYP1A1 enzymatic activity in LNCaP cells (24, 25). However, the mechanism by which testosterone represses TCDD-induced transcription remains unclear. The present study was undertaken to get further insight into the mechanism of the inhibitory effect of testosterone. In a previous study (24), the inhibitory effect of testosterone was evaluated only on the CYP1A1 gene in LNCaP cells. AR expressed in LNCaP cells has a mutation (Thr877 to Ala in ligand-binding domain). In the present study, we used DHT as an androgen and assayed the inhibitory effects of DHT against CYP1A2, CYP1B1, and AhRR genes, typical activated AhR-regulated genes, in addition to CYP1A1. T47D breast cancer cells were also used because they express wild type AR. DHT repressed 3MC-induced transcription with all four genes in both LNCaP and T47D cells (Fig. 1). These results suggest that repression of AhR-regulated gene transcription by DHT is not due to mutant AR and a general phenomenon in AhR- and AR-positive cells. While AhRR functions to repress the transcriptional activity of AhR (18), AhRR mRNA levels were actually reduced in 3MC/DHT-treated cells (Fig. 1), indicating that AhRR is not involved in the inhibitory effect of DHT.

It is possible that activated AR or other transcription factors bind to *cis*-elements, except for XRE, present in the transcriptional regulatory regions of these AhR-

regulated genes to suppress the transcriptional activation. The inhibitory effect of DHT was observed in the XRE-driven luciferase reporter assay in this study (Fig. 2). Furthermore, similar effects were observed in a luciferase reporter assay in COS cells transfected with vectors expressing human AR and AhR (31). These results indicate that the presence of the XRE is sufficient for DHT to inhibit AhR-activated transcription. The inhibitory effects of DHT on 3MC-induced CYP1 family mRNA levels and XRE-driven luciferase activity are more pronounced in LNCaP cells than in T47D cells. Therefore, we used LNCaP cells in the subsequent experiments.

We verified that AR is involved in the repression of 3MC-induced transcription by DHT using the AR knockdown technique with AR siRNA (Fig. 3).

While the reduction in AhR and/or Arnt protein levels would lead to decreased induction of AhR-regulated genes, DHT did not alter the cellular AhR and Arnt protein levels in 3MC-treatment (Fig. 4).

It is needless to say that activated AhR must be translocated from the cytoplasm to the nucleus and then bind to DNA in order to activate the transcription. So, we examined the effect of DHT on translocation of AhR to the nucleus. As shown in Fig. 5A, DHT did not affect the translocation of AhR to the nucleus. Then, we examined whether complex formation between AR and AhR was altered by DHT in the nucleus. DHT significantly facilitated the formation of a complex between AR and AhR in the nucleus of 3MC-treated cells (Fig. 5: B and C), and these results suggest that complex formation between AR and AhR plays an important role in the repression of 3MC-induced transcription of CYP1 family genes by DHT. We have obtained some results showing that activated AhR binds to unliganded AR and causes translocation of AR to the nucleus from the cytoplasm (manuscript in preparation). Activated AhR had a similar effect in LNCaP cells (30). So, AR protein is detected in the nuclear extracts and in the immunoprecipitates of AhR from the 3MC-treated cells (Fig. 5B).

We propose two hypotheses to account for the molecular mechanism of the suppressive effects of DHT that we have reported here: 1) AhR in a complex with AR has decreased binding affinity for the XRE. This suggestion is based on the observation that AR and c-Jun form a complex that has reduced DNA binding ability and results in mutual repression of AR- and AP1-regulated gene transcription (34). Arnt is supposed to play an important role in binding of AhR/Arnt heterodimer to DNA since the CAC half-site of the core sequence of XRE (CACGC) was expected to be recognized by Arnt (35, 36). If AR inhibits the dimerization of AhR with Arnt by complex formation between

AR and AhR, the binding of AhR to the XRE is repressed. 2) After complex formation between AR and AhR and XRE binding, we hypothesize that recruitment of coactivators and the formation of the transcriptional machinery is inhibited due to steric hindrance caused by AR bound to AhR.

In conclusion, DHT represses 3MC-induced transcription of AhR-regulated genes, CYP1A1, CYP1A2, CYP1B1, and AhRR in LNCaP and T47D cells. While complex formation between AR and AhR was observed with both activated and unliganded AR, the complex was enriched in the nuclear extracts of 3MC/DHT-treated cells compared to 3MC-treated cells. These results suggest that DHT suppresses 3MC-induced transcription of AhR-regulated genes by facilitating complex formation between AR and AhR that results in reduced transcriptional activity.

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