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Ligand-independent androgen receptor antagonism caused by the newly developed pesticide pyrifluquinazon (PFQ)

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ABSTRACT

Androgen receptor (AR) is an essential component to activate AR dependent gene transcriptions. Despite wide acceptance of pharmacological controls on transcriptional pathway depending on competitive inhibitions of ligand binding, only a few examples, AR antagonism *via* ligand-independent mechanisms, have been recognized. Pyrifluquinazon(PFQ), a newly developed pesticide, induced representative AR antagonism against rats in *in vivo* and *in vitro*. Intriguingly, this AR antagonism was not based on inhibition of ligand binding. Instead, the evidence suggested that the AR antagonism was induced as a consequence of decline of cellular AR protein level. This study demonstrated that AR N-terminal region could be an essential element for a ligand-independent mechanism underling the AR antagonism by PFQ. Our findings should provide a novel insight into the regulation of AR-mediated transcription.

1. Introduction

Steroid hormones play important roles in the development of secondary sex characteristics in vertebrate and invertebrate species *via* binding to the nuclear receptors. Ligand binding to the androgen receptor (AR) initiates an array of responses leading to AR-dependent gene transcription and, as such, AR is a target for pharmacological regulation of AR-mediated pathways. Flutamide and bicaltamide, non-steroidal competitive ligand binding inhibitors that have been utilized therapeutically for prostate cancers, efficiently suppress AR mediated transactivation *via* inhibition of ligand binding [1–3]. Some environmental contaminants, including pesticides, may be antiandrogenic *via* competitive inhibition of ligand binding of AR [4,5] and pose a hazard to human health.

In the absence of ligand, AR function can be modulated by ligand-independent pathways through recruitment of accessory proteins or co-chaperones [6–8]. Despite this potential activity in the regulation of AR function, only a few examples of antiandrogenic effects have been recognized as being independent of ligand binding through non-competitive mechanism. In this study, we present evidence for ligand-independent disruption of androgen signaling *via* a decrease in cellular AR protein content, for the new insecticide PFQ (1,1,3,4-tetrahydro-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]quinazolin-2,4-dione).

2. Materials and methods

2.1. Chemicals

PFQ (>98%) was synthesized at Nihon Nohyaku Co., Ltd (Tokyo, Japan). Hydroxyflutamide and dihydrotestosterone (DHT) were purchased from LKT Labs, Inc. (St. Paul, MN) and Wako Chemicals (Osaka, Japan), respectively. Antibodies for AR (N-20) and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma (St. Louis, MO), respectively.

2.2. Animal protocols

Male Sprague-Dawley rats (SPF) from Japan SLC Inc. (Slc:SD) were housed in fiber reinforced plastics cages and acclimated to controlled ambient conditions (22 ± 3 °C, 50 ± 30% humidity and 12 h light–dark cycle). Animals were provided with MR diet (Nohsan Cooperation) and tap water *ad libitum*. The diet was periodically checked for bacterial contamination. Tap water was filtered through 5 μm pore filters to remove undissolved residues. Animal rooms were ventilated with HEPA-filtered fresh air 15 times per day. All animal handlings were in accordance with the guidelines for proper conduct of animal experiments (Science Council of Japan) and approved by the animal welfare committee of Nihon Nohyaku Co., Ltd. The use of ether anesthesia, although understood not to be a best-practice for animal protocols, was consistent with some studies [9,10].

2.3. Cell culture

The human kidney cell line HEK293 and human breast cancer cell line MDA-kb2 were purchased from American Type Culture Collection (Manassas, VA). These

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cell lines were maintained according to the supplier's instructions, except for use of Dulbecco's modified Eagle's medium (DMEM) for HEK293 cell cultures.

2.4. Plasmids

The rat AR cDNA (GenBankTM accession number M23264) was amplified through two sequential nested PCRs from rat liver Marathon-ready cDNA (Clontech Laboratories, San Jose, CA) using specific primers to introduce restriction enzyme sites (*Not1*, *BamH1* and *Kaz*) and a typical Kozak sequence. The rat AR cDNA generated by RT-PCR was subcloned into a pCR2.1-TOPO vector through TA-cloning according to the manufacturer's instructions (Invitrogen, Carlsbad, CA.). The resultant bacterial clones were digested at restriction sites (*Not1*. *BamH1*) and inserted into a pcDNA3.1. The rARΔ641-902, a mutant sequence devoid of the entire ligand-binding domain (LBD) and C-terminal region, was amplified by PCR from the rat AR cDNA as the template, as described elsewhere [11]. The pcDNA3.1 plasmids ligated with full-length rat AR and rARΔ641-902 cDNA were subcloned into a pcDNA3.1-Myc-His and used as the expression vector in the transcriptional assays described in Section 2.5. Luciferase reporter vector was constructed through insertion of ARE2-TATA-Luc sequence into the pGL4.14-basic vector largely based on Moilanen et al. [12] with a minor modulation. First, double strand oligonucleotide of TATA sequence including *HindIII* restriction enzyme site (from adenovirous E1b) was inserted between *HindIII* restriction enzyme site of pGL4.14[luc/hygro] vector (promega). Subsequently, to introduce ARE tandem sequence into upstream of TATA sequence, a double strand oligonucleotide including *SacI* restriction enzyme site, which consisted of sense-primer (5′-cATAGTACGTGATGTTCTAGGCCTAGTACGTGAT GTTCTCgagct-3′) and antisense primer (5′-cGAGAACATCACGTACTAG GCCTAGAACATCACGTACTATgagct-3′) was inserted between Sac I restriction enzyme site of pGL4.14-TATA vector. The pGL4.14-ARE-TATA vector thus obtained was used for AR luciferase reporter vector.

2.5. Transactivation assay

For transactivation assays, the HEK293 cells and the MDA-kb2 cells were seeded on 12-well plates in phenol red-free DMEM medium supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum, and Lebovitz's L-15 medium, respectively. For the transactivation assay of rat AR, HEK293 cells were co-transfected with the rAR expression vector (0.8 μg/well) and pGL4.14-ARE2-TATA-Luc (0.4 μg/well), using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. After a 24 h transfection, medium was replaced with fresh medium containing DHT or PFQ (added as an ethanolic solution to give a final concentration of 0.1%), followed by a 10 h exposure. The assays with MDA-kb2 were performed as an earlier report except for a 20 h exposure with DHT or PFQ [13]. Luciferase activities were determined with Luciferase Assay Systems (Promega, Madison, WI) according to the manufacturer's instruction. Luciferase light units were converted to the relative-hold induction and normalized with the induction achieved with 0.2 nM DHT based on solvent control values. To monitor non-specific cytotoxicity, pGL4.50[*luc2*/CMV/Hydro] (Promega, Madison, WI) (0.5 μg/well) was transfected to HEK293 cell and luciferase activity was determined in the absence of DHT exposure.

2.6. Western blot analysis

Cells were lysed in passive lysis buffer (Promega, Madison, WI), separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with 1000-fold-diluted primary antibodies against AR (N-20) for 2 h at room temperature, and then with secondary antibodies for 1 h at room temperature. Detection and quantification of the target protein were carried out with ECL plus (GE Healthcare, Tokyo, Japan). α-Tubulin was used as an internal reference for the normalization of cellular AR protein content.

2.7. Equilibrium ^{3}H-R1881 binding assay with rat prostate AR

An equilibrium binding assays using ^{3}H-R1881 were performed as described previously [14], with minor modifications as follows. Rat ventral prostate tissues were collected from 10-week-old Slc:SD rats 24 h after castration and homogenized in TEDG [14]. The supernatant containing cytosolic AR was obtained by centrifugations (30,000 × *g*, 30 min, 4 °C) and stored at −80 °C until use. For equilibration of ligand binding, the cytosol, diluted to 20-fold volume was incubated with ^{3}H-R1881 (2 nM or 10 nM) and compounds for 12–16 h at 4 °C. For determination of non-specific binding, 10 μM of radio-inert DHT was added into the mixture at the start of the incubation. Radioactivity bound to the receptor was precipitated with hydroxyapatite (BIO-RAD Laboratories, Hercules, CA) and centrifugation. The hydroxyapatite precipitant was washed 3 times with buffer (40 mM Tris–HCl, pH7.5, 100 mM KCl) and radioactivity determination with liquid scintillation counting [14]. The Kd value of ^{3}H-R1881 determined through the procedure described above was 0.93 nM which was nicely consistent to the reported values (0.685–1.57 nM) [14].

2.8. Real time RT-PCR

Ventral prostates collected from intact rats were homogenized in Sepasol RNAI (Nakarai tesque, Inc.) and deproteinated by chloroform extraction. Total RNA precipitated with isopropanol was reversely transcribed to cDNA using PrimeScript reagent Kit (Takara Bio.). AR mRNA was amplified with primers 5′-CACCATGCAACTTCTTCAGCA-3′ (forward) and 5′-CGAATTGCCCCCTAGGTAACT-3′ (reverse). The reaction mixture composed of 2 μl of the reverse-transcriptional product, the primers above described and SYBR Premix Ex TaqTM (Perfect Real Time, Takara Bio) was analyzed by Takara PCR Thermal Cycles. The values were normalized with β-actin mRNA and expressed as percentile toward vehicle control.

2.9. Hershberger assay

The Hershberger assay was performed according to conventional protocol [15]. Male SPF rats on postnatal day (PND) 35 were acclimated under the same experimental condition as described in Section 2.2. After 1-week acclimation (PND 42), rats were castrated under anesthetization with diethyl-ether vapor. Prior to dosing, rats were assigned to each dosing group to minimize inter-group differences in means of body weight. At 7 days after castration (PND 49), they were dosed with PFQ at 100 or 200 mg/kg/day, or vehicle as a negative control and 5- or 10 mg/kg flutamide as a positive control, started and repeated during 10 days with gavage. Each dose group consisted of 6 animals. Dosing suspensions of PFQ and flutamide were freshly prepared with corn oil to give a dosing volume of 5 ml/kg. Concurrent with PFQ and flutamide exposure, DHT was subcutaneously injected at 1.25 mg/kg/day for 10 days. On the day after the final dosing (PND 60), male accessory organs including ventral prostate, combined seminal vesicles plus coagulating gland (SVCG) and levator ani muscle and bulbocavernous muscle (LABC) were collected and weighed.

2.10. Single oral dosing on rat prostatic AR protein and AR mRNA

Male SPF rats (Slc:SD) on PND 63 were dosed with PFQ at 100- or 200 mg/kg (or vehicle) with gavage after 1 week acclimation under same experimental condition as described in Section 2.2. Each dosing group consisted of 4 animals. Simultaneously, flutamide was dosed at 5 mg/kg as reference compound. The dosing suspensions were prepared as described in Section 2.9. Prior to dosing, rats in each dosing group were fasted for 16 h. At 6-, 12-, and 24 h after dosing, the rats were euthanized by exsanguination under ether and ventral prostates were extirpated. These tissues were washed and trimmed off excess fat in ice-cold saline followed by weighing. The prostates were cut in half along central axis; one half was used to determine AR protein and the other half was used to determine AR mRNA. Samples were stored at −80 °C until use.

For determination of rAR protein, the samples were homogenized with a Teflon pestle in 10 volume lysis buffer (Promega, Madison, WI) on wet ice. Homogenates were centrifuged at 14,000 × *g*, 4 °C for 20 min and the supernatant was diluted with an equal volume of Laemmli sample buffer (BIO-RAD, CA.) and denatured in boiling water for 5 min. Samples were subjected to SDS-agarose gel electrophoresis (ePAGEL 10%, ATTO) for western blot analysis as described in Section 2.6. For determination of rAR mRNA, the rat ventral prostate collected from intact rats were homogenized in Sepasol RNAI (Nakarai tesque, Inc.) and deproteinated with chloroform. Real time RT-PCR was performed as described in Section 2.8.

Statistical significance determined by Dunnett's test is indicated by asterisks as follows: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. Transcriptional assay employing rat AR (rAR)

Transactivation assays, such as a reporter gene assay to quantify AR dependent gene expression, have been utilized as a sensitive tool to detect functional modulations of AR. The transactivation assay employing rAR in HEK293 cells clearly indicated that PFQ inhibited DHT-dependent transactivation mediated by rAR in a concentration-dependent manner (IC_{50} = 7.2 μM, Fig. 1A). PFQ had no effect on the constitutive luciferase activity induced by the CMV promoter (Fig. 1B). As such, inhibition of the DHT dependent transactivity by the compound was attributed to disruption of AR mediated signaling pathway rather than inhibition of luciferase activity or promoter activity.

We examined AR protein content in the samples exposed to PFQ. Cellular AR protein in the same cell culture system used for the transactivation assay was determined by Western blotting (Fig. 1C). PFQ exposure significantly decreased the intracellular rAR