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Anti-androgen effects of the pyrethroid pesticide cypermethrin on interactions of androgen receptor with corepressors

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ABSTRACT

To clarify whether the mechanism of androgen receptor (AR) antagonism of the pyrethroid pesticide cypermethrin associates with the interactions between the AR and corepressors silencing mediator for thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR), we have developed the mammalian two-hybrid assays. The AR N-terminal domain 1–660 amino acid residues were subcloned into the plasmid pVP16 to construct VP16-ARNTD. The C-terminal receptor interaction domains (RIDs) of SMRT and NCoR were used to construct pM-SMRT and pM-NCoR. The constructed vectors pVP16-ARNTD, pM-SMRT or pM-NCoR, the reporter pG5CAT and the control pCMVβ were cotranfected into the CV-1 cells. The cells were treated with cypermethrin at the indicated concentrations. The AR N terminus interacted with RIDs of SMRT and NCoR. The interactions between the AR and corepressors SMRT and NCoR were enhanced by cypermethrin, and the significant enhancement was detected at the concentration of 10^{-5} M. The mammalian two-hybrid assays demonstrate the utility to detect the interactions of the AR with SMRT and NCoR. Cypermethrin functions as an anti-androgen by enhancing the associations of the AR with SMRT and NCoR. We provide a novel mechanism in anti-androgen action of cypermethrin associated with the recruitment of SMRT and NCoR to AR.

1. Introduction

There is growing interest in the possible adverse health effects caused by endocrine-disrupting chemicals (EDCs), which can mimic or block transcriptional activation elicited by naturally circulating steroid hormones (Kumar et al., 2008; Diamanti-Kandarakis et al., 2009). EDCs pose a significant challenge to the society, the health of humans and the environment (Schug et al., 2011; Zoeller et al., 2012; Rotroff et al., 2013). The evidences indicate EDCs may contribute to male reproductive impairments including decline in the human sperm count, poor semen quality and reduced male fertility (Swan et al., 2003; Meeker et al., 2004; Swan, 2006). Furthermore, the studies support the hypothesis that EDCs exposure influences the risk of hormonally mediated cancers including breast, prostate and testicular cancers (Martin et al., 2008; Prins, 2008; Cohn, 2011). A huge number of pesticides including pyrethroid pesticides have been identified as EDCs, which provide a potential threat to the human and wildlife (Andersen et al., 2002; Kojima et al., 2004; Lemaire et al., 2006).

The EDCs are commonly thought to exert action as receptor agonists or antagonists primarily through direct interaction with nuclear hormone receptors including estrogen receptors (ERs), androgen receptors (ARs), and thyroid receptors (TRs) (Tabb and Blumberg, 2006; Ding et al., 2010). Many pesticides in current use to control agricultural and indoor pests have been revealed as AR antagonists by interacting with the AR to interfere with AR signaling pathway (Kelce et al., 1995; Andersen et al., 2002; Kojima et al., 2004; Martin et al., 2008; Orton et al., 2011). We have focused on the anti-androgen activity and the mechanisms of the EDCs for many years (Xu et al., 2005, 2006, 2008; Hu et al., 2012; Pan et al., 2012). Our studies have demonstrated some pyrethroid pesticides including cypermethrin act as AR antagonists by binding to the AR and inhibiting subsequent gene expression in the AR reporter gene assay (Xu et al., 2006, 2008). Because androgens and AR mediate a wide range of developmental and physiological responses in the male, the pesticides as AR antagonists will have a greater impact on male developmental programming and reproductive tract maturation. The data have shown that the widely used pyrethroid pesticide cypermethrin is associated with certain male reproductive damages (Elbetieha et al., 2001; Wang et al., 2010, 2011).

Cypermethrin is among the EDCs with identified anti-androgen activity which may target the AR signaling to prevent transcriptional initiation. Therefore, the down-regulation of AR signaling

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may be involved in anti-androgen action of cypermethrin. However, the molecular mechanisms are not clear. Then our studies have revealed the underlying mechanisms of the antagonistic effect of cypermethrin on AR-mediated action. We have shown cypermethrin can inhibit the amino- and carboxyl-terminal (N/C) interaction of the AR using the mammalian two-hybrid assay (Hu et al., 2012). Our data also have demonstrated cypermethrin inhibits AR activity by disrupting the interaction between the AR activation function-1 (AF-1) and steroid receptor coactivator-1 (SRC-1). Cypermethrin prevents the coactivator SRC-1 recruitment to AR, which eventually leads to repression of the AR-dependent genes expression (Pan et al., 2012).

In addition to the mechanisms associated with AR N/C interaction and SRC-1 recruitment, recruitment of corepressors seems also to play important role in the anti-androgen action. The data have shown that the transcriptional activity of AR is modulated by the corepressors in addition to the coactivators (Lin et al., 2002; Liao et al., 2003; Hodgson et al., 2008). Corepressors are factors that associate with AR and repress its transcriptional activity. The two corepressors including silencing mediator for retinoid and thyroid hormone receptors (SMRT) and the nuclear receptor corepressor (NCoR) have been shown to play key roles in transcriptional repression of AR (Cheng et al., 2002; Liao et al., 2003; Yoon and Wong, 2006). The N-CoR and SMRT binding to the AR are thought to recruit histone deacetylase complex (HDAC) in the AR complex to reduce the level of histone acetylation and repress transcription of AR target genes (Guenther et al., 2001). The data confirm the role of endogenous SMRT and NCoR as negative regulators of AR activity to contribute to the inhibitory functions of AR. Reducing the recruitment of corepressors to AR favors an agonist response, whereas increasing the recruitment favors an antagonist response. Therefore, enhancement of the corepressors recruitment to the AR may block AR signaling and contribute to AR antagonist activity, which will reveal a new mechanism for AR antagonism.

AR antagonists are commonly used in prostate cancer therapy to inactivate the transcriptional activity of the AR and then to inhibit the proliferation of prostate cancer. The clinically used AR antagonist cyproterone acetate (CPA) mediates the inhibitory function on AR through recruitment of SMRT to the AR (Dotzlaw et al., 2002). Bicalutamide, which is widely used for prostate cancer treatment, can mediate the recruitment of NCoR to the androgen-regulated PSA gene (Chen et al., 2004). Mifepristone is a potent antagonist of glucocorticoid as well as progesterone receptors. It has been demonstrated that mifepristone functions as a potent anti-androgen with minor agonistic activity (Song et al., 2004). The AR–NCoR interaction can be markedly enhanced by mifepristone (Hodgson et al., 2005). The data confirm that SMRT and NCoR interact with the AR and contribute to antagonistic activity of clinically used anti-androgens. Enhancement of corepressor recruitment to the AR may be a new mechanism of the anti-androgens. However, there is lacking of data regarding the antagonistic mechanisms of the environmental anti-androgens involving the role of corepressors. We hypothesize that the anti-androgen cypermethrin may also enhance SMRT and NCoR recruitment to AR and therefore function as AR antagonist, which may be an underlying novel mechanism of cypermethrin.

In the present study we have developed the mammalian two-hybrid assays to evaluate the interaction between the AR and corepressors SMRT and NCoR. For better understanding of the molecular basis of anti-androgen action of cypermethrin, we used the assays to examine the effect of cypermethrin on the corepressors recruitment to the AR. We show that cypermethrin functions as an AR antagonist by enhancing the associations of the AR with corepressors SMRT and NCoR. The study provides a new insight into anti-androgen action of cypermethrin, with a significant role for the corepressors SMRT and NCoR.

2. Materials and methods

2.1. Reagents

The chemical cypermethrin was purchased from Sigma–Aldrich (St. Louis, MO, USA) with the purity of 99%. The chemical was dissolved in absolute ethanol at a concentration of 10^{-2} M, stored at −20 °C. The chemical was diluted to desired concentrations in phenol red-free RPMI1640 medium (Sigma Chemical Co.) immediately before use. The final ethanol concentrations in the culture medium did not exceed 0.1%, and this concentration did not affect cell yields. The ESCORT V Transfection Reagent was from Sigma–Aldrich (St. Louis, MO, USA). The chloramphenicol acetyl transferase enzyme-linked immunosorbent assay (CAT-ELISA) kit was from Roche Molecular Bioch (Mannheim, Germany). The β-galactosidase (β-Gal) Enzyme Assay System with reporter lysis buffer was purchased from Promega (Madison, WI, USA).

2.2. Plasmids

The Mammalian Matchmaker™ Two-Hybrid Assay Kit was obtained from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The pG5CAT was used as the reporter vector which contains the CAT gene downstream of five consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. To construct the fusion vector pVP16-ARNTD, the AR N-terminal regions encoding 1–660 amino acid residues were fused to the VP16 activation-domain (AD) in the pVP16 vector. To generate the fusion vector pM-ARLBD, the AR C-terminal regions encoding 624–919 amino acids were cloned into the GAL4 DNA-binding domain (DBD) in the pM vector. The fusion vector pM-SMRT was generated by fusing the C-terminal domains encoding 2101–2400 amino acids of SMRT to the pM vector, and the fusion vector pVP16-SMRT was generated by fusing the same regions to the pVP16 vector, respectively. The C-terminal domains encoding 1926–2435 amino acids of NCoR were cloned into the pM vector to give pM-NCoR, and the same domains were cloned into the pVP16 vector to give pVP16-NCoR, respectively. The β-gal expression plasmid, pCMVβ, was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA), and used as an internal control for transfection efficiency.

2.3. Cell culture and transfection

The CV-1 cell line was purchased from Institute of Biochemistry and Cell Biology in Shanghai, Chinese Academy of Science (Shanghai, China). The CV-1 cells were maintained in phenol red-free RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere in 5% CO_2 at 37 °C. The cells were plated in triplicate in six-well microtiter plates (Nunc, Denmark) at a density of about 1.0×10^5 cells per well with 2 ml RPMI1640 medium containing 10% charcoal-dextran stripped FBS allowed to incubate 24 h. The cells were cotransfected with the reporter vector pG5CAT, pVP16-ARNTD or pM-ARLBD, pM-SMRT or pVP16-SMRT or pM-NCoR or pVP16-NCoR and the internal control vector pCMVβ using the ESCORT V Transfection Reagent. After an incubation period of 24 h, the cells were then treated with cypermethrin at the indicated concentration. The medium with 0.1% ethanol was used as a vehicle control. To avoid cytotoxicity caused by the tested chemical, the concentrations were performed at $\leq 10^{-5}$ M. After incubation for 24 h, the cells were lysed and then subjected to the assays for CAT and β-Gal amounts.

2.4. Reporter gene assays

The cells were harvested 24 h after dosing. After washed three times with phosphate-buffered saline (PBS, pH 7.4), the cells were lysed with 400 μl 1 × lysis buffer (Promega, Madison, WI, USA) per well. After centrifuged at 12,000 × *g* for 5 min to remove debris, the cell lysates were either analyzed immediately or quick-frozen at −80 °C. The CAT amounts were measured with the commercial CAT-ELISA kit following the manufacturer's instructions. Additional aliquots of cell lysates were assayed to determine β-Gal amounts using the β-Gal Enzyme Assay System with Reporter Lysis Buffer. The CAT amounts were normalized to the β-gal amounts. The relative CAT amounts were presented as fold induction which is calculated relative to the untreated control vector.

2.5. Statistical analysis

All experiments were carried out in triplicate wells and repeated at least three times. The results were expressed as mean ± S.D. from triplicate wells for each dose. One-way analysis of variance (ANOVA) was used for multiple comparisons, followed by Dunnett's *t*-test for multiple comparisons with controls. Statistical significance was considered when $p < 0.05$.

3. Results

3.1. Interactions of the AR N terminus with corepressors SMRT and NCoR

In this study, the mammalian two-hybrid assays were developed to detect the interactions of the AR with corepressors SMRT