Toxicology and Applied Pharmacology 272 (2013) 453–464

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity

Lisbeth Stigaard Kjeldsen, Mandana Ghisari, Eva Cecilie Bonefeld-Jørgensen *

Centre for Arctic Health, Department of Public Health, Aarhus University, Aarhus, Denmark

ARTICLE INFO

Article history:
Received 2 May 2013
Revised 11 June 2013
Accepted 25 June 2013
Available online 16 July 2013

Keywords:
Pesticide
Endocrine disruptor
Estrogenic
Antiandrogenic
Luciferase reporter gene assay
Aromatase

ABSTRACT

The endocrine-disrupting potential of pesticides is of health concern, since they are found ubiquitously in the environment and in food items. We investigated *in vitro* effects on estrogen receptor (ER) and androgen receptor (AR) transactivity, and aromatase enzyme activity, of the following pesticides: 2-methyl-4-chlorophenoxyacetic acid (MCPA), terbuthylazine, iodosulfuron-methyl-sodium, mesosulfuron-methyl, metsulfuron-methyl, chlormequat chloride, bitertanol, propiconazole, prothioconazole, mancozeb, cypermethrin, tau fluvalinate, malathion and the metabolite ethylene thiourea (ETU). The pesticides were analyzed alone and in selected mixtures. Effects of the pesticides on ER and AR function were assessed in human breast carcinoma MVLN cells and hamster ovary CHO-K1 cells, respectively, using luciferase reporter gene assays. Effects on aromatase enzyme activity were analyzed in human choriocarcinoma JEG-3 cells, employing the classical $[^3H]_2O$ method.
Five pesticides (terbuthylazine, propiconazole, prothioconazole, cypermethrin and malathion) weakly induced the ER transactivity, and three pesticides (bitertanol, propiconazole and mancozeb) antagonized the AR activity in a concentration-dependent manner. Three pesticides (terbuthylazine, propiconazole and prothioconazole) weakly induced the aromatase activity. In addition, two mixtures, consisting of three pesticides (bitertanol, propiconazole, cypermethrin) and five pesticides (terbuthylazine, bitertanol, propiconazole, cypermethrin, malathion), respectively, induced the ER transactivity and aromatase activity, and additively antagonized the AR transactivity.
In conclusion, our data suggest that currently used pesticides possess endocrine-disrupting potential *in vitro* which can be mediated via ER, AR and aromatase activities. The observed mixture effects emphasize the importance of considering the combined action of pesticides in order to assure proper estimations of related health effect risks.

Introduction

Exogenous compounds that alter the normal functioning of the endocrine system are called endocrine-disrupting chemicals (EDCs), referring to their potential to cause adverse health effects in humans and wildlife. Based on several *in vitro* and *in vivo* studies, a considerable number of environmental contaminants have been identified as EDCs and among them are several pesticides (Andersen et al., 2002, 2008; Birkhoj et al., 2004; Grunfeld and Bonefeld-Jorgensen, 2004; Hofmeister and Bonefeld-Jorgensen, 2004; Long et al., 2003; Nellemann et al., 2003; Vinggaard et al., 2005a, 2005b; Vinggaard et al., 2002).

The endocrine-disrupting (ED) potential of many pesticides is of health concern, because their residues are found not only in the environment but also in many food items as a result of the ubiquitous nature of pesticide usage with minimal precautions. Therefore, humans are exposed to pesticides via various routes throughout life, including dietary and environmental exposure (water, soil, air) and also through occupational settings. Several studies have shown the presence of pesticide residues and their metabolites in human tissues, demonstrating a global distribution (Arrebola et al., 2012; Jaga and Dharmani, 2003; Sanghi et al., 2003; Zietz et al., 2008).

In particular, the activity of estrogen-mimicking compounds has been under investigation for the last decades. Studies have demonstrated that a broad range of pesticides possess estrogenicity *in vivo*, e.g., methoxychlor, kepone and dichlorodiphenyltrichloroethane (DDT), and/or *in vitro*, e.g., *o,p′*-DDT, *p,p′*-DDT, endosulfan, toxaphene, dieldrin, methiocarb, prochloraz, fenarimol, triadimefon and triadimenol (Andersen et al., 2002; Bonefeld Jorgensen et al., 1997, 2005 Grunfeld

Abbreviations: 4-AOD, 4-Androsten-4-ol-3,17-dione; AR, androgen receptor; CA, concentration addition; CV, coefficient of variation; CYP, cytochrome P450; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; DHT, dihydrotestosterone; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; ED, endocrine-disrupting/endocrine disruption; EDC, endocrine-disrupting chemical; ER, estrogen receptor; ETU, ethylene thiourea; HF, hydroxyflutamide; MCPA, 2-methyl-4-chlorophenoxyacetic acid; QSAR, quantitative structure–activity relationship; R1881, methyltrienolone; SC, Solvent control; SD, Standard deviation.

* Corresponding author at: Centre for Arctic Health & Unit of Cellular and Molecular Toxicology, Department of Public Health, Aarhus University, Bartholins Allé 2, Building 1260, DK-8000 Aarhus C, Denmark. Fax: +45 8716 7307.
E-mail address: ebj@mil.au.dk (E.C. Bonefeld-Jørgensen).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.06.028

and Bonefeld-Jorgensen, 2004; Soto et al., 1994). Estrogens are sex hormones of great importance for the female reproductive system. Moreover, they play key roles in both male and female fetal development and are essential for growth, differentiation and function of a broad range of tissues, e.g. the central nervous system, the musculoskeletal system, the immune system and the cardiovascular system (Bjornstrom and Sjoberg, 2005; Heldring et al., 2007). Thus, any disruption of estrogen receptor (ER) signaling pathways can contribute to adverse health effects such as infertility, developmental abnormalities and endocrine-related cancer (Bonefeld-Jorgensen et al., 2011; Mueller, 2004; Sikka and Wang, 2008).

Studies have shown that various pesticides, such as vinclozolin, dichlorodiphenyldichloroethylene (*p,p′*-DDE), prochloraz, procymidone, linuron, methoxychlor, dieldrin and fenitrothion, exert antiandrogenic effects by interfering with the androgen receptor (AR) (Andersen et al., 2002; Luccio-Camelo and Prins, 2011; Vinggaard et al., 2002). The AR is the key regulatory element of androgen cell signaling. Physiologically, AR-regulated gene expression is responsible for male sexual differentiation *in utero* and male reproductive function and development, including spermatogenesis (Dehm and Tindall, 2007; Gao et al., 2005).

Adverse trends in male reproductive health have appeared during the last decades (Andersson et al., 2008; Carlsen et al., 1992), and a synchronized increase in the incidence of male reproductive problems (e.g. testicular cancer, genital abnormalities, reduced semen quality and subfertility) has been hypothesized (Skakkebaek, 2003; Skakkebaek et al., 2001). Several studies have indicated a correlation between pesticide exposure and reduced male fertility (Lifeng et al., 2006; Oliva et al., 2001; Padungtod et al., 2000; Swan et al., 2003) and female fertility (Abell et al., 2000). Moreover, epidemiological studies have suggested a link between environmental exposure to pesticides and the risk of hormone-related cancer such as breast cancer and prostate cancer (Mnif et al., 2011; Sankpal et al., 2012).

There is a special concern regarding ED effects on growth and development of the embryo/fetus, being sensitive to hormonal fluctuations (Bigsby et al., 1999; Skakkebaek et al., 2001; Swan et al., 2005). Exposure to EDCs during early life stages is suspected to cause irreversible damage and developmental abnormalities, which may not be manifested until later in life. It is assumed that fetuses (i.e., pregnant women) and children are especially vulnerable groups concerning pesticide exposure. Studies have indicated an impaired reproductive development, including an increased prevalence of cryptorchidism, in sons of women working as gardeners or living on farms where pesticides have been used (Andersen et al., 2008; Carbone et al., 2007; Komarek et al., 2010; Kristensen et al., 1997; Weidner et al., 1998; Wohlfahrt-Veje et al., 2011).

So far, most pesticide research has been focusing on persistent organochlorine insecticides (e.g., DDT, chlordane and dieldrin) due to their former extensive use and bioaccumulative properties. However, during the last decades their use has been largely superseded by pesticides which do not persist appreciably in the environment. Therefore, it is imperative to further investigate the potential impact on human health of currently used "non-persistent" pesticides.

The present study was part of the cross-disciplinary research project HOPE, aiming for assessment of the ED potential of currently used pesticides in Denmark from different pesticide groups: phenoxy acids, triazines, sulfonylureas, growth regulators, azoles, dithiocarbamates, pyrethroids and organophosphates (Table 1). A dithiocarbamate metabolite was included in the study as well, because the mother compound, mancozeb, decomposes when exposed to moisture and air and has low persistence in soils (Komarek et al., 2010). Apart from the organophosphate insecticide malathion (banned in Denmark since 2008 but included in the study because of an extensive Danish fruit import) and the dithiocarbamate metabolite, the test compounds represent active ingredients in pesticide formulations commonly used in Denmark today.

The test compounds were selected on the basis of (*i*) application of pesticide, (*ii*) amount of pesticide (in kg) used in Denmark in 2006, (*iii*) acreage (in hectares) treated with pesticide in Denmark, and (*iv*) knowledge from published evidence of ED effects of pesticides and ongoing pesticide research (based on an extensive survey of EU assessment reports and additional literature search).

Herbicides such as the phenoxy acids, triazines and sulfonylureas are generally used to reduce weeds in various agricultural and horticultural settings. The phenoxy acids are post-emergence growth inhibitors applied to protect crops such as cereal grains, corn, fruit trees and some vegetables (Barr and Needham, 2002; Santilio et al., 2009). The triazines are broad-spectrum pre- and post-emergence herbicides used to control broadleaf and grassy weeds in various crops such as corn, sorghum, sugar cane etc. and similarly, sulfonylurea herbicides are used to control weeds in a variety of crops and vegetables (Barr and Needham, 2002; Sarmah and Sabadie, 2002). Plant growth regulators are important components in agricultural production as well. In grain crops, these compounds are used to reduce the length and strengthen the straw in order to prevent lodging and thus reduce the risk of difficulties during harvesting (Sorensen et al., 2009).

Various pesticides have been described for their activity against a broad range of fungi and insects. The azoles and dithiocarbamates are extensively used in plant protection worldwide due to their antimycotic properties. As the organochlorine pesticides were phased out in most countries, alternative insecticides such as the organophosphates and pyrethroids have become widely used to control insect pests in a variety of crops. Exposure to synthetic pyrethroids is expected to increase among the general population due to regulatory restrictions on the use of common organophosphates in the USA and other countries (Meeker et al., 2008).

The specific objectives of the present study were to evaluate *in vitro* the effect of the pesticides (Table 1) on ER and AR function as well as aromatase activity. The aromatase, encoded by the *CYP19*, is the key enzyme in the biosynthesis of estrogens from cholesterol, since it catalyzes the final rate-limiting step in which androgens are converted to estrogens. Consequently, the aromatase enzyme is crucial for the maintenance of the homeostatic balance between estrogen and androgen sex hormones. Aromatase activity (i.e., regulation of estrogen synthesis from androgens) is thought to be a critical endpoint concerning sexual development and differentiation (Jones et al., 2006).

As humans are continuously exposed to multiple EDCs simultaneously, a growing concern regarding the risk of mixture effects has emerged. Several *in vitro* and *in vivo* studies have demonstrated mixture effects of pesticides (Birkhoj et al., 2004; Hass et al., 2012; Jacobsen et al., 2012; Kjaerstad et al., 2010a; Nellemann et al., 2003) and other EDCs such as plasticizers and phenols (Ghisari and Bonefeld-Jorgensen, 2009; Kruger et al., 2008). In addition to the single pesticides, we also analyzed two mixtures selected on the basis of observed *in vitro* and *in silico* potencies of the single test compounds in the HOPE project, including analyses of hormone receptor interference, aromatase enzyme activity, steroid hormone synthesis and quantitative structure–activity relationship (QSAR).

Materials and methods

Chemicals. The high-affinity ER ligand 17β-estradiol (E2) was obtained from Sigma-Aldrich (Denmark) and used as positive dose–response control in the ER transactivation assay. E2 was dissolved in 96% extra pure ethanol from Merck (Darmstadt, Germany) to produce a 100 nM stock solution.

The AR agonists, methyltrienolone (R1881) and dihydrotestosterone (DHT) were obtained from Perkin Elmer (Hvidovre, Denmark) and Sigma-Aldrich (Denmark), respectively. The AR antagonist hydroxyflutamide (HF) was from MikroMol GmbH (Luckenwalde, Germany). R1881, DHT and HF were used as dose–response controls in the AR transactivation assay. R1881 and HF were dissolved in 96%