Molecular and Cellular Endocrinology 392 (2014) 90–105

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Isomer-nonspecific action of dichlorodiphenyltrichloroethane on aryl hydrocarbon receptor and G-protein-coupled receptor 30 intracellular signaling in apoptotic neuronal cells

M. Kajta [a,*], E. Litwa [a], J. Rzemieniec [a], A. Wnuk [a], W. Lason [a], A. Zelek-Molik [b], I. Nalepa [b], M. Grzegorzewska-Hiczwa [c], K. Tokarski [c], A. Golas [d], E. Guzik [e], A. Grochowalski [f], K.A. Szychowski [g], A.K. Wojtowicz [a,g]

[a] Department of Experimental Neuroendocrinology, Institute of Pharmacology, Polish Academy of Sciences, 12 Smetna Street, 31-343 Krakow, Poland
[b] Department of Brain Biochemistry, Institute of Pharmacology, Polish Academy of Sciences, 12 Smetna Street, 31-343 Krakow, Poland
[c] Department of Physiology, Institute of Pharmacology, Polish Academy of Sciences, 12 Smetna Street, 31-343 Krakow, Poland
[d] Department of Genetics and Evolution, Institute of Zoology, Jagiellonian University, 9 Gronostajowa Street, 30-387 Krakow, Poland
[e] Department of Cell Biology and Imaging, Confocal Microscopy Laboratory, Institute of Zoology, Jagiellonian University, 9 Gronostajowa Street, 30-387 Krakow, Poland
[f] Department of Analytical Chemistry, University of Technology, 24 Warszawska Street, 31-155 Krakow, Poland
[g] Laboratory of Genomics and Biotechnology, University of Agriculture, 1B Rędzina Street, 30-274 Krakow, Poland

ARTICLE INFO

Article history:
Received 20 February 2014
Received in revised form 28 April 2014
Accepted 9 May 2014
Available online 21 May 2014

Keywords:
GSK-3β
Bcl-2
Caspases
Estrogen receptors
Neurotoxicity
Primary neuronal cell cultures

ABSTRACT

Extended residual persistence of the pesticide dichlorodiphenyltrichloroethane (DDT) raises concerns about its long-term neurotoxic effects. Little is known, however, about DDT toxicity during the early stages of neural development. This study demonstrated that DDT-induced apoptosis of mouse embryonic neuronal cells is a caspase-9-, caspase-3-, and GSK-3β-dependent process, which involves *p,p'*-DDT-specific impairment of classical ERs. It also provided evidence for DDT-isomer-nonspecific alterations of AhR- and GPR30-mediated intracellular signaling, including changes in the levels of the receptor and receptor-regulated mRNAs, and also changes in the protein levels of the receptors. DDT-induced stimulation of AhR-signaling and reduction of GPR30-signaling were verified using selective ligands and specific siRNAs. Co-localization of the receptors was demonstrated with confocal microscopy, and the presence of functional GPR30 was detected by electrophysiology. This study demonstrates that stimulation of AhR-signaling and impairment of GPR30-signaling play important roles in the propagation of DDT-induced apoptosis during the early stages of neural development.

1. Introduction

Dichlorodiphenyltrichloroethane (DDT) is a synthetic pesticide that was banned for agricultural use worldwide since 1970's. The Stockholm Convention on Persistent Organic Pollutants applies to DDT which is scheduled for eventual elimination. Nevertheless, its limited use continues, mainly to fight the spread of malaria due to targeting the voltage-gated sodium channels on the insect neurons

Abbreviations: Ac-DEVD-*p*NA, N-acetyl-asp-glu-val-asp *p*-nitro-anilide; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DDT, dichlorodiphenyltrichloroethane; DIV, day *in vitro*; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; G1, (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone: a selective GPR30 agonist; G15, 3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline: a selective GPR30 antagonist; GPR30, G-protein-coupled receptor 30; GSK-3β, glycogen synthase kinase 3β; *Hprt*, hypoxanthine phosphoribosyltransferase coding gene; ICI 182,780, 7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl) sulfinyl]nonyl]estra-1,3,5 (10)-triene-3,17-diol; LDH, lactate dehydrogenase; MPP, methyl-piperidino-pyrazole: a selective ERα antagonist; PHTPP, 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol: a selective ERβ antagonist; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; SB 216763, 3-(2,4-Dichlorophenyl)-4-(1-methyl-1Hindol-3-yl)-1H-pyrrole-2,5-dione: GSK-3β inhibitor; sEPSC, spontaneous excitatory postsynaptic current; WB, Western blotting; Z-LEHD-FMK, Z-Leu-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone trifluoroacetate salt hydrate: caspase-9 inhibitor; Z-LETD-FMK, Z-Leu-Glu(OMe)-Thr-Asp(OMe) fluoromethyl ketone: caspase-8 inhibitor.

* Corresponding author. Tel.: +48 12 6374022; fax: +48 12 6374500.
E-mail address: kajta@if-pan.krakow.pl (M. Kajta).

http://dx.doi.org/10.1016/j.mce.2014.05.008
0303-7207/

(Narahashi, 2000). DDT consists of a mixture of *p,p'*-DDT (77–85%) and *o,p'*-DDT (15–23%) which persist in the environment and accumulate in the food chain and tissues of living organisms. Immunization against DDT reduces the uptake of the pesticide in adipose tissue, blood, spleen and brain (Hrafnkelsdottir et al., 2007). Although DDT exerts low acute toxicity, its extended residual persistence raises concerns about long-term adverse effects, and it is still the subject of ongoing studies. Exposure to DDT was found to be associated with alterations in dopaminergic neurotransmission (Faro et al., 2009), which could result in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. Recent epidemiological study exhibited the association between the incidence of neural degenerations and exposure to pesticides, e.g. organochlorine endosulfan, organophosphate chlorpyrifos, dicarboximide vinclozolin, bipyridyl paraquat (Parron et al., 2011). Colborn (2004) pointed to an increased risk of deficit and hyperactivity disorder, autism, and associated neurodevelopmental problems in parallel with increasing exposure to contaminants. Children and adults chronically exposed to DDT had overall poorer performance on tests of verbal attention and eye-hand coordination (Van Wendel de Joode et al., 2001; Rocha-Amador et al., 2009). Neonatal exposure to the pesticide may increase adult susceptibility to neurotoxins and impair the faculties of learning and memory (Eriksson and Talts, 2000). It has also been postulated that low doses of DDT may have epigenetic effects, causing incomplete methylation of specific gene regions in the young brain (Shutoh et al., 2009). Little is known, however, about DDT toxicity during the early stages of neural development.

DDT is an environmental contaminant that exerts estrogenic actions through binding to classical estrogen receptors (ERs). The subsequent transcription of estrogen-responsive genes disrupts endocrine homeostasis in the organism. The World Health Organization has declared the field of endocrine disruption a high research priority (IPCS/WHO, 2002). The most estrogenic component of DDT seems to be *o,p'*-DDT exhibiting 100-fold greater binding affinity to ERs than *p,p'*-DDT (Kojima et al., 2004). In addition, the pesticide may alter growth factor signaling and interrupt cross-talk between the aryl hydrocarbon receptor (AhR) and the transcriptional activity of ERs. However, AhR participation in DDT-induced effects is unclear and seems to depend on the type of tissue affected by the pesticide (Jeong and Kim, 2002; Rasier et al., 2008; Wojtowicz et al., 2011). In addition, little is known about DDT interaction with the newly identified membrane ER, G-protein-coupled receptor 30 (GPR30; also known as G-protein-coupled ER 1), which mediates non-genomic estrogen actions such as the inhibition of oxidative stress-induced apoptosis, up-regulation of NGF, and effects on the EGF/ERK/c-Jun apoptotic pathway (Prossnitz and Barton, 2011; Chimento et al., 2010).

Our previous study provided evidence for the involvement of classical ER signaling in the attenuation of AhR-mediated apoptosis in mouse cerebral neurons (Kajta et al., 2009). We also demonstrated a key role of GPR30 in the neuroprotective action of phytoestrogen daidzein (Kajta et al., 2013). The aim of the present study was to investigate the mechanisms of apoptotic and neurotoxic actions of DDT isomers on mouse embryonic neuronal cells, especially their possible interactions with AhR- and GPR30-mediated intracellular signaling pathways. The recently available GPR30-selective antagonist (G15) and agonist (G1) were used as pharmacological tools to differentiate the functions of classical ERs and the membrane ER GPR30 in the estrogen pathway. Furthermore, a widely used high affinity estrogen receptor antagonist, ICI 182,780, appeared to possess properties of the GPR30 agonist (Thomas et al., 2005).

In the present study, we evaluated *p,p'*-DDT- and *o,p'*-DDT-induced activation of caspase-3 and lactate dehydrogenase (LDH) release in mouse neuronal cells in primary cultures. These data were supported by Hoechst 33342 and calcein AM staining to visualize apoptotic nuclei and cell survival, respectively. To assess whether DDT exhibited tissue-dependent effects, we studied the effects of DDT in hippocampal, neocortical, and cerebellar tissues. Specific inhibitors of caspase-8, caspase-9, and glycogen synthase kinase-3β (GSK-3β) were employed to indicate which of these enzymes participated in DDT-induced caspase-3-dependent apoptosis. The involvement of classical ERs in DDT actions was verified using selective ERα and ERβ antagonists, in addition to an ER antagonist with affinity for both ERα and ERβ. DDT-induced alterations of AhR- and GPR30-signaling pathways were studied with selective ligands and specific siRNAs, and the presence of functional GPR30 in mouse embryonic hippocampal neurons was detected by electrophysiology. The levels of specific mRNAs and proteins were measured with RT-PCR, Western blottings (WBs) and ELISAs, and the cellular distributions of AhR and GPR30 were demonstrated with a confocal microscope.

2. Materials and methods

2.1. Materials

α-naphtoflavone, β-naphtoflavone, Ac-DEVD-*p*NA (N-acetyl-asp-glu-val-asp *p*-nitro-anilide), anti-β-actin antibody, BSA (bovine serum albumin), $CaCl_2$, DMSO (dimethyl sulfoxide), poly-ornithine, EGTA, glucose, GSK-3β, caspase-9 and caspase-8 inhibitors (SB 216763, Z-Leu-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone trifluoroacetate salt hydrate, Z-Leu-Glu(OMe)-Thr-Asp(OMe) fluoromethyl ketone), EDTA-free protease inhibitors, HEPES, KCl, K-gluconate, $MgCl_2$, NaCl, NaOH, octylphenoxypolyethoxyethanol (IGEPAL CA-630), poly-ornithine, sodium ATP, sodium deoxycholate, sodium GTP, SDS and Tween 20 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Alexa 488-conjugated anti-goat IgG, calcein AM, and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR, USA). The B27 and neurobasal medium were obtained from Gibco (Grand Island, NY, USA), and Bradford reagent was obtained from Bio-Rad Laboratories (Munchen, Germany). The cDNA reverse transcription kit, RNAlater, TaqMan Gene Expression Master Mix and TaqMan probes for the specific genes coding the receptors AhR, ERα, ERβ, and GPR30, the receptor target genes *Cyp1a1*, *Cyp19a*, and *c-fos*, and the apoptosis-related genes *Gsk-3b*, and *bcl-2* were obtained from Life Technologies Applied Biosystems (Carlsbad, California, USA), culture plates were obtained from TPP Techno Plastic Products AG (Trasadingen, Switzerland), and PVDF membranes were received from Merck Millipore (Billerica, MA, USA). The Cy3-conjugated anti-rabbit IgG was obtained from Jackson Immunoresearch Laboratories Inc (West Grove, PA, USA), and the BM Chemiluminescence Blotting Substrate, Cytotoxicity Detection Kit, Lysis Buffer (4.5 M guanidine-HCl, 100 mM sodium phosphate, pH 6.6) and mRNA isolation Kit were obtained from Roche Diagnostics GmbH (Mannheim, Germany). G1 ((±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benz odioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone), G15 (3a*S**, 4*R**,9b*R**)-4-(6-Bromo-1,3-ben zodioxol-5-yl)-3a,4,5,9b-3*H*-cyclope nta[*c*]quinoline), ICI 182,780 (7α,17β-[9-[(4,4,5,5,5-Pent afluoropentyl) sulfinyl]nonyl]estra-1,3,5 (10)-trene-3,17-diol), MPP (methyl-piperidino-pyrazole), and PHTPP (4-[2-Phenyl-5,7-bis (trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol), were purchased from Tocris Bioscience (Bristol, UK). Donkey anti-goat IgG, donkey anti-rabbit IgG, goat polyclonal anti-AhR antibody (sc-8088), rabbit polyclonal anti-AhR antibody (sc-5579), rabbit polyclonal anti-Bcl-2 antibody (sc-492), rabbit polyclonal anti-ERα antibody (sc-7207), rabbit polyclonal anti-ERβ antibody (sc-8974), rabbit polyclonal anti-GPR30 antibody (sc-134576), rabbit polyclonal anti-GSK-3β antibody (sc-9166), siRNA AhR (sc-72178), and