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ARYL HYDROCARBON RECEPTOR-MEDIATED APOPTOSIS OF NEURONAL CELLS: A POSSIBLE INTERACTION WITH ESTROGEN RECEPTOR SIGNALING

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Abstract—Activation of aryl hydrocarbon receptors (AhRs) induces neuronal damage, but the mechanism by which this occurs is largely unknown. This study evaluated the effects of an AhR agonist, β-naphthoflavone, on apoptotic pathways in mouse primary neuronal cell cultures. β-Naphthoflavone (0.1–100 μM) enhanced caspase-3 activity and lactate dehydrogenase (LDH) release in neocortical and hippocampal cells. These data were supported at the cellular level with Hoechst 33342 and calcein AM staining. α-Naphthoflavone inhibited the action of β-naphthoflavone, thus confirming specific activation of AhRs. A high-affinity estrogen receptor (ER) antagonist, ICI 182,780, and a selective estrogen receptor modulator (SERM), tamoxifen, enhanced β-naphthoflavone-mediated apoptosis. Another SERM, raloxifene, and an ERα antagonist, methyl-piperidino-pyrazole, did not affect β-naphthoflavone-induced caspase-3 activity. However, they inhibited β-naphthoflavone-induced LDH release at a late hour of treatment, thus suggesting delayed control of AhR-mediated neuronal cell death. The apoptotic effects of β-naphthoflavone were accompanied by increased levels of AhRs, and these receptors colocalized with ERβ as demonstrated by confocal microscopy. These data strongly support apoptotic effects of AhR activation in neocortical and hippocampal tissues. Moreover, this study provides evidence for direct interaction of the AhR-mediated apoptotic pathway with estrogen receptor signaling, which provides insight into new strategies to treat or prevent AhR-mediated neurotoxicity. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: Ac-DEVD-*p*NA, N-acetyl-asp-glu-val-asp-*p*-nitro-anilide; AhR, aryl hydrocarbon receptor; DIV, day/days *in vitro*; DMSO, dimethyl sulfoxide; ECL, chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERE, estrogen response element; GFAP, glial fibrillary acidic protein; LDH, lactate dehydrogenase; MPP, methyl-piperidino-pyrazole; NAD, nicotinamide adenine dinucleotide; PBS, phosphate-buffered saline; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol; RT, room temperature; SERM, selective estrogen receptor modulator; TBS, Tris-buffered saline; TCDD, tetrachlorodibenzo-*p*-dioxin; U, the enzymatic unit of lactate dehydrogenase activity; ZVAD-FMK, fluoromethylketone.

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The majority of toxic effects of dioxins are mediated by the aryl hydrocarbon receptor (AhR). Therefore, this receptor may be responsible for dioxin intoxication, which creates severe clinical problems, such as behavioral and cognitive impairments and an increased number of newborns with deformed brains (Eriksson and Talts, 2000). AhRs are present in many tissues including the brain, where their distribution pattern in the preoptic area closely overlaps that of GABAergic neurons (Hays et al., 2002). AhR is a ligand-dependent transcription factor that activates transcription of genes, such as: CYP1A1, CYP1A2, and CYP1B1, and oncogenes (Kawajiri and Fujii-Kuriyama, 2007). Tryptophan and its metabolites, bilirubin, 7-ketocholesterol and lipoxin A4 were proposed to be endogenous ligands for AhR (Denison and Nagy, 2003). However, no endogenous ligand with high affinity for AhR has been yet identified.

The molecular mechanism underlying AhR-induced neurotoxicity is unknown. Thus, AhR-mediated effects in the nervous system are related mainly to necrosis. Nayar et al. (2002) demonstrated that rat prenatal exposure to AhR agonist tetrachlorodibenzo-*p*-dioxin (TCDD) resulted in neurodevelopmental deficits, possibly due to altered activity of Sp1 transcription factor, which prevents oxidative stress-induced neuronal cell death. Kuchiiwa et al. (2002) found that, in mouse, administration of TCDD 8 weeks prior to pregnancy resulted in 50–75% decrease in serotonergic neurons in the raphe nuclei. In addition, Kakeyama et al. (2001) and Cho et al. (2002) found altered expression of NMDA receptor subunits (an ionotropic receptor for glutamate named after its selective agonist *N*-methyl-D-aspartic acid) after treating animals or neocortical cell cultures with TCDD, which promote neuronal necrosis. Little is known, however, about apoptotic effects mediated by AhR. This is particularly important because apoptosis occurs at each stage of neurodevelopment but may also be attributed to neurodegenerative diseases. There are few data involving AhRs in brain apoptosis (Tillitt and Papoulias, 2002; Dong et al., 2002, 2004). Recently, it has become evident that AhRs may also be involved in neural development, likely through interaction with Wnt (Wingless-type) signaling (Chesire et al., 2004; Gordon and Nusse, 2006), in addition to mediating neuronal cell death in response to environmental pollutants.

No effective therapy against AhR-mediated neurotoxicity has been established yet. Some studies suggest an-

tioxidants, such as vitamin E and dihydroascorbic acid, to effectively protect human epithelial cells from TCDD toxicity (Hirai et al., 2002). Others propose protection by resveratrol, which shows antagonist activity against the AhR (Amakura et al., 2003). Protective properties could also involve estrogens and their receptors, which are important regulators of apoptosis, especially that mediated by the mitochondrial pathway (Kajta and Beyer, 2003). Those protective effects are expressed against the Bcl-2-induced apoptosis in Alzheimer's and Parkinson's diseases. According to recent observations, estrogens and their receptors play also an important role in neurogenesis (Perez-Martin et al., 2003). Estradiol-17β was found to suppress apoptosis in the developing cerebral cortex by decreasing the cell-surface expression of the Fas receptor in neuroblasts (Cheema et al., 2004). Furthermore, it became evident that estrogen receptor (ER) β is crucial for brain development as evidenced by developmental abnormalities in the brains of ERβ knockout mice (Wang et al., 2003). Recent findings suggest that ERβ in the embryonic brain is necessary for the development of calretinin-immunoreactive GABAergic interneurons and for neuronal migration in the cortex (Fan et al., 2006). Because estrogens have protective effects, defining the interaction between ER- and AhR-mediated signaling in neuronal cells could provide insight into a new protective strategy against AhR-induced apoptosis in the developing brain.

Based upon neuroprotection attributed to estrogens, we hypothesized that AhR-induced apoptosis might be counteracted by ER signaling. Our previous study provided evidence for an interaction of AhR signaling with the anti-apoptotic action of genistein, a plant-derived isoflavone that binds to ERs with SERM (selective estrogen receptor modulator) properties (Kajta et al., 2007). Because β-naphthoflavone is an AhR agonist, we evaluated its impact on apoptotic processes in the mouse primary neuronal cell cultures. In order to verify whether AhR-mediated effects were tissue- and age-dependent, we related them to neocortical and hippocampal tissues, both on 1 and 7 days *in vitro* (DIV). In addition to ER antagonist- and SERM-mediated effects on β-naphthoflavone-induced caspase-3 activity and lactate dehydrogenase (LDH) release, the interaction between AhR-induced apoptosis and ER signaling was evaluated by determining the levels and cellular distribution of AhR and ERβ.

EXPERIMENTAL PROCEDURES

Materials

α-Naphthoflavone, β-naphthoflavone, N-acetyl-asp-glu-val-asp-*p*-nitro-anilide (Ac-DEVD-*p*NA), anti-β-actin mouse monoclonal antibody (A5316), anti–glial fibrillary acidic protein (GFAP), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), poly-ornithine, sodium dodecyl sulfate (SDS), Triton X-100, and Tris buffered saline (TBS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Active caspase-3 enzyme-linked immunosorbent assay (ELISA) Kit came from Calbiochem (EMD Chemicals, affiliate of Merck KGaA, Darmstadt, Germany). Alexa 488–conjugated anti-goat IgG came from Invitrogen (Carlsbad, CA, USA), 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). Bradford reagent came from Bio Rad Laboratories (Munchen, Germany), the Cy3-conjugated anti-rabbit IgG came from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA), and the Cytotoxicity Detection Kit was from Roche Diagnostics GmbH (Mannheim, Germany). Goat polyclonal anti-AhR antibody (sc-8088), horseradish peroxidase (HRP)–conjugated IgG (sc-2004), rabbit polyclonal anti-ERβ antibody (sc-8974), and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). ICI 182,780, MPP (methyl-piperidino-pyrazole), PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol), raloxifene, and tamoxifen were purchased from Tocris Bioscience (Bristol, UK), and culture plates came from Nunc A/S (Roskilde, Denmark).

Primary neocortical and hippocampal cell cultures

Neocortical and hippocampal tissues for primary cultures were prepared from Swiss mouse embryos at 15–17 days of gestation and were cultivated essentially as described (Junghans and Kappler, 1999; Kajta et al., 2007). Animal care followed official governmental guidelines and all efforts were made to minimize the number of animals used and their suffering. All procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were granted an approval from the Bioethics Commission as compliant with Polish Law (21 August 1997). Cells were suspended in estrogen-free neurobasal medium supplemented with B27, which is recommended for primary neuronal cultures, and plated at a density of 2.5×10^5 cells per cm^2 onto poly-ornithine (0.01 mg per ml)-coated multi-well plates. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 1 or 7 days prior to experimentation and the medium was changed every other day after plating. Additionally, the culture medium was changed prior to treating cultures with all compounds selected for the study. The level of astrocytes, as determined by the content of intermediate filament protein GFAP, was up to 10% for all cultures (Kajta et al., 2004).

Treatment

Primary neocortical and hippocampal cell cultures were exposed to β-naphthoflavone (0.1, 1, 10, 100 μM) for 6 h or 24 h. Specificity of AhR activation was examined using the receptor antagonist α-naphthoflavone (1 μM), which may also partially inhibit the aryl hydrocarbon hydroxylase, CYP1A1, an enzyme that metabolizes steroids, fatty acids, and xenobiotics. The involvement of ER signaling in β-naphthoflavone-mediated effects was verified with the high affinity ER antagonist, ICI 182,780, SERMs tamoxifen and raloxifene, ERα antagonist MPP, and ERβ antagonist PHTPP. PHTPP was used only to determine possible effects of ERβ on the level of the active form of caspase-3. ICI 182,780 binds to ERα and ERβ with similar affinity and antagonizes their functions (Tremblay et al., 1997; Leblanc et al., 2007; Jiang et al., 2008; Traynor et al., 2008). It is devoid of any partial agonism both *in vitro* and *in vivo*. ICI 182,780 has a steroidal structure that competitively binds to the ER with an affinity much greater than that of tamoxifen or raloxifene. MPP is selective, high affinity antagonist at ERα. It displays about 200-fold selectivity for ERα over ERβ (Sun et al., 2002). PHTPP is selective ERβ antagonist that displays 36-fold selectivity over ERα (Tocris Bioscience Catalogue 2008). All these compounds were used at a concentration of 1 μM and added to culture media 45–60 min before β-naphthoflavone was added. These agents, except for water soluble MPP, were originally dissolved in DMSO and then further diluted in culture medium to result in DMSO concentrations below 0.1%.

Identification of apoptotic cells

Apoptotic cells were detected by Hoechst 33342-staining 48 h after initial treatment, as described previously (Kajta et al., 2007).