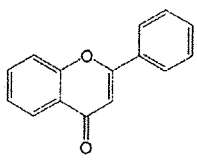
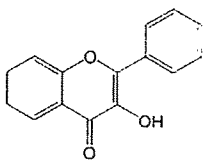
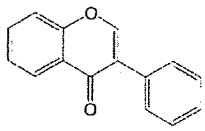
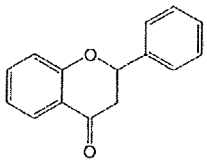
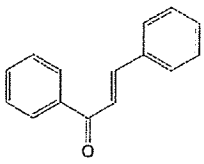
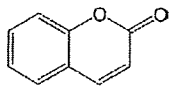
AhR, which is a ligand-activated transcription factor that stimulates gene expression when coupled with AhR nuclear translocator (ARNT) (Carver and Bradfield, 1997; Denison and Whitlock, 1995). TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a ligand of AhR) induces CYP1A1, CYP1A2, and CYP1B1 that hydroxylate E2. Moreover, TCDD and other agonists for AhR disrupt the transcriptional activity of ER by degradation of ER (Buchanan et al., 2000, 2002). Thus, agonists of AhR may mimic the effects of estrogen through the mechanism that is involved in the degradation of ER by a transcriptional active AhR–ARNT complex (Ohtake et al., 2003).

In this study, we investigated the effect of flavonoids on AhR and ER action (Table 1), and found that in MCF-7 cells, 2-phenylchromone (FLA, the structurally most basic compound) inhibited the expression of ER target genes, suggesting antagonist activity of ERα expressed via AhR in an indirect manner.

Table 1
Structures of flavonoids used in this study

Structures	Classifications	Test chemicals
	Flavones	Flavone (FLA)
	Flavonols	Kaempferol Quercetin
	Isoflavones	Biochanin A Daidzein Genistein
	Flavanones	Hesperetin Hesperidin Naringenin
	Chalcones	Phloretin
	Coumarins	Bromofluoro coumarin 2-Bromomethyl-7-methoxycoumarin Coumestrol 4-Methylumbeliferone

Materials and methods

Chemicals

FLA, E2, kaempferol, hesperidin, and α- and β-naphthoflavone (α-NF and β-NF) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), hesperetin and bromofluoro coumarin were from Extrasynthese (Genay Cedex, France), 2-bromomethyl-7-methoxycoumarin was from BD Gentest (CA, USA), 4-methylumbeliferone was from Sigma-Aldrich Co. (MO, USA), and MG-132 was from Calbiochem (Darmstadt, Germany). Biochanin A, coumestrol, daidzein, genistein, naringenin, phloretin, and quercetin were provided by Dr. H. Utsumi (Kyushu University).

Yeast assay for AhR ligand activity

The yeast transformed with the AhR–ARNT complex and xenobiotic-responsive element (XRE) plasmids was used as described by Miller (Miller, 1999). The AhR ligand activity was determined essentially according to the method of Adachi et al. (2001). The yeast strain YCM3 was grown for 5 h at 30 °C in SD medium lacking tryptophan. Test chemicals were added at given concentrations to 5 μl of culture and 200 μl of SD medium containing 2% galactosidase and incubated overnight at 30 °C. After the cell density was determined by reading O.D. at 595 nm, 10 μl of cell suspension was added to 140 μl of *Z*-buffer and β-galactosidase activity was determined by *o*-nitrophenol-β-D-galactopyranoside for 60 min at 37 °C. Absorbance was read at 415 nm.

Cells

MCF-7 cells were grown for routine maintenance in Eagle's minimal essential medium (EMEM) with phenol red (Nissui Pharmaceuticals Co., Tokyo, Japan), supplemented with 10 mM non-essential amino acids (Nacalai Tesque Co., Tokyo, Japan) and 10% dextran-charcoal treated fetal bovine serum (FBS). Cells were maintained in a humidified environment at 37 °C with 5% CO_2 in air.

RNA isolation and RT-PCR

MCF-7 (4×10^5 cells/ml) cells were plated in 35-mm dishes and, after 48 h incubation, treated with chemicals for 24 h. After treatment, the cells were washed twice with PBS and RNA was then isolated using Trizol (Invitrogen, CA). cDNA synthesized from 0.8 μg of total RNA using ReverTra Ace-α™ (TOYOBO, Osaka, Japan) and PCR for progesterone receptor (PR), pS2, CYP1A1, and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) was performed using Ampli Taq (Roche, Basel, Switzerland). The optimum cycle number that fell within the exponential range of response was used for PR (30 cycles), pS2 (21 cycles), CYP1A1 (30 cycles), or G3PDH (17 cycles).

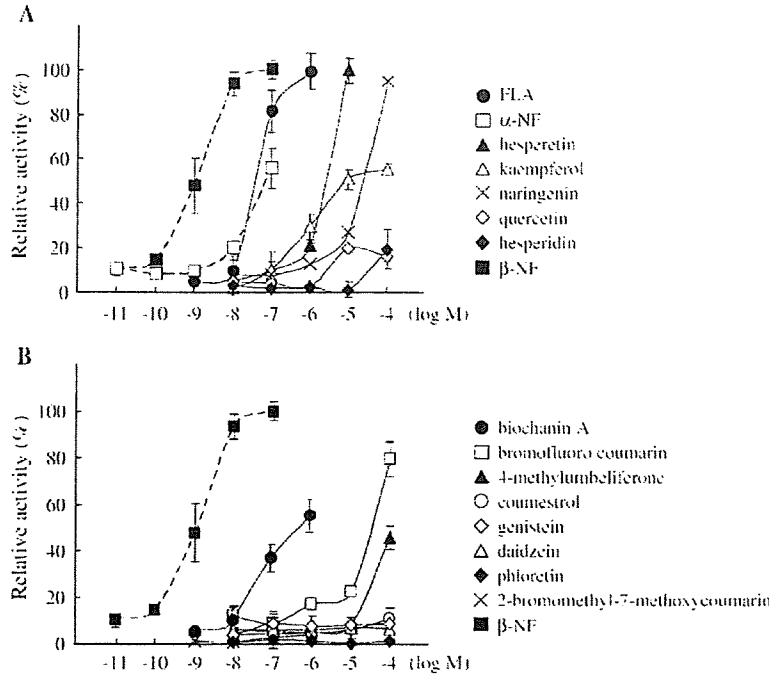


Fig. 1. AhR agonist activity of flavonoids. The test chemicals were applied to the recombinant yeast assay for AhR ligand activity as described in Materials and methods. The test chemicals were divided into two groups; a group included flavones, flavonols, and flavanones (A), and the other group included isoflavones, chalcones, and coumarins (B). Some flavonoids including FLA showed positive activity.

Yeast two-hybrid assay (ER)

We used a yeast two-hybrid assay system with the rat ER (rER) α and the coactivator, TIF2 as described in earlier works (Nishihara et al., 2000; Jung et al., 2004).

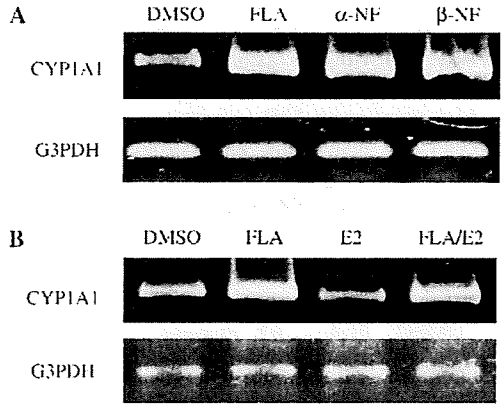


Fig. 2. Effect of FLA on the expression of CYP1A1 mRNA in MCF-7 cells. FLA (10 μM) was incubated with MCF-7 cells for 24 h in the absence (A) and presence (B) of E2 (10 pM). α- and -β NFs (10 μM) were used as positive controls, and DMSO as negative control. The expression of CYP1A1 and G3PDH mRNA (as an internal control) was detected by RT-PCR as described in Materials and Methods. FLA induced the expression as well as α-, β-NFs (A), but it was inhibited by E2 (B).

Estrogen receptor competitive binding assay

The binding activity of chemicals to human ER (hER) α was determined using a fluorescence polarization assay by FP Screen-for-Competitors Kit (ERα, high sensitivity; PanVera, Madison, WI). Briefly, 1 μl of each chemical solution was added to 49 μl of screening buffer in tubes and mixed well by shaking. Then, 50 μl of ERα-fluorescence estrogen (ES1) complex solution was added to the tube, incubated at room temperature for 1 h, and the fluorescence was determined using BEACON2000 (PanVera, Madison, WI). DMSO (0% inhibition) instead of the chemical solution was used as a negative control and 10 μl of ES1 (50 nM) instead of ERα ES1 complex as a

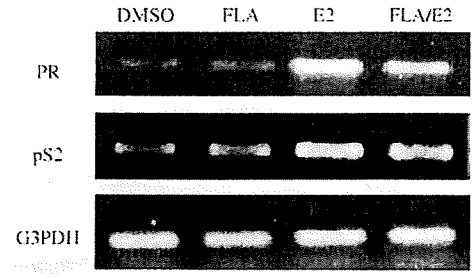


Fig. 3. Effect of FLA on mRNA level of E2-dependent target genes. FLA (10 μM) was incubated with MCF-7 cells for 24 h in the absence and presence of E2 (10 pM). The expression of PR, pS2 and G3PDH (as an internal control) mRNA was detected by RT-PCR. FLA repressed the expression by E2.

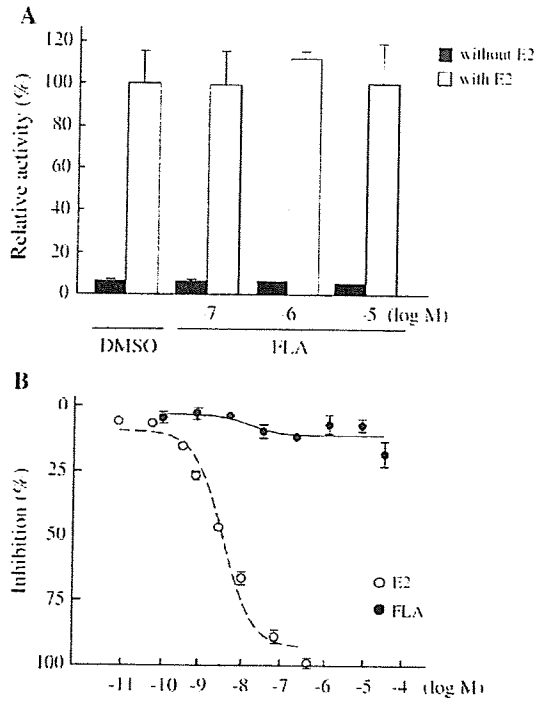


Fig. 4. Estrogen activity of FLA. (A) ERα agonist or antagonist activity of FLA was examined by yeast two-hybrid assay, and the relative activity (%) was calculated as the percentage of E2 (5 nM) activity (100%). (B) Binding affinity of FLA was examined by ERα competitive binding assay as described in Materials and Methods. FLA exhibited no effect on estrogen binding activity to ERα.

positive control (100% inhibition). Curve fitting was performed using GraphPad Prism 2.01 software to obtain IC_{50}.

Protein isolation and Western blots

MCF-7 (4×10^5 cells/ml) cells were plated in 35-mm dishes and, after 48 h, treated with chemicals for the indicated times. After treatment, the cells were washed twice with PBS and then lysed in 70 μl of lysis buffer containing 8 M urea, 1% NP-40, and 2% 2-mercaptoethanol. After removing the cell debris, the supernatants were used for protein concentration assay. The protein was boiled for 2 min, resolved on a 10% SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore immobilon transfer membrane, MA). After the membrane was blocked using 3% skimmed milk (Yukijirushi, Tokyo, Japan) overnight at 4 °C, it was probed with primary antibodies ERα (1:200 in 1% skimmed milk, Santa Cruz Biotechnology Inc., CA) and G3PDH monoclonal antibodies (1:1000 in 1% skimmed milk, Chemicon International, MA). Following incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Amersham Pharmacia Biotech, UK).

Results and discussion

In the yeast reporter gene assay, the effect of flavonoids on AhR was evaluated because it had been reported that TCDD and other AhR agonists inhibited the expression of several E2-induced genes without binding to ER (Buchanan et al., 2002). Among 14 tested flavonoids, 7 compounds (FLA, biochanin A, hesperitin, kaempferol, naringenin, bromofluoro coumarin and 4-methylumbeliferone) dose-dependently increased the AhR activity (Fig. 1A and B). On the contrary, Hamada et al. (2006) reported that some of these flavonoids suppressed TCDD-induced CYP1A1 expression in dioxin-responsive HepG2 cells by permeating Caco-2 cell monolayers. The difference may involve the metabolism of flavonoids by drug-metabolizing enzymes in the Caco-2 cells. Furthermore, we investigated FLA, which was particularly responsible for the high activity of AhR. Although FLA was ten times weaker than β-NF (positive control), it was stronger than the other flavonoids and α-NF (a second positive control). Therefore, FLA was chosen for further study. AhR agonists induce the expression of several genes. For example, mRNA levels of CYP1A1 are induced by TCDD and other AhR ligands (Whitlock et al., 1996). RT-PCR assay indicated that in MCF-7 cells, FLA (fold, 2.6±1.0) induced the expression of CYP1A1 mRNA at similar levels to α- and β-NF (folds, 2.2±0.5 and 2.4±0.5) (Fig. 2A). In contrast, E2 inhibits the expression of genes induced by AhR ligands (Stacey et al., 1999). In this study, the induction of CYP1A1 mRNA by FLA (fold, 2.2±0.1) was inhibited in combination with E2 (fold, 1.5±0.4) (Fig. 2B). The expression of G3PDH mRNA was measured as control, and then it was not changed. Thus, the results suggest that FLA acted as an AhR agonist.

When we tested the anti-estrogenic activity of many chemicals including flavonoids by the yeast two-hybrid assay and the competitive ER binding assay, some flavonoids and

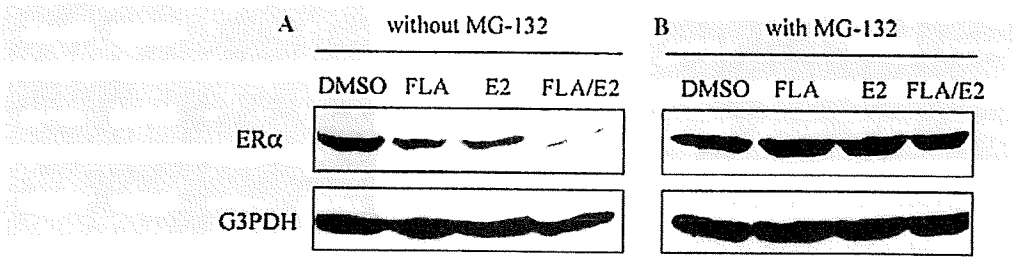


Fig. 5. Effect of FLA on ERα protein level. In the absence (A) or presence (B) of MG-132 (protease inhibitor), FLA (10 μM) was incubated with/without E2 (10 pM) in MCF-7 cells for 24 h. The cell lysates were subjected to SDS-PAGE and Western blot analysis for ERα and G3PDH (as an internal control). FLA induced the degradation of ERα and it was prevented by MG-132.

their metabolites were determined to be ER antagonists as well as agonists (Nishihara et al., 2000; Ahn et al., 2004a,b; Okamoto et al., 2006). As mentioned earlier in this work, however, these assays are insufficient to explain the anti-estrogenic activity of some flavonoids because these assays can measure only when chemicals directly affect the interaction with ERα. When we screened for the active compound by the reporter gene assay using MCF-7 cells in the presence of E2, some chemicals inhibited the transcriptional activity of ER (details not shown). The expressions of PR and pS2 mRNA were induced by E2 depending on the dose, indicating that these expressions demonstrated estrogen activity (Seo et al., 2003; Petz et al., 2002; Kim et al., 2000). As an AhR agonist, FLA was investigated for estrogen action. E2 (1 pM) induces the mRNA expression of PR (fold, 9.6±1.2) and pS2 (fold, 6.3±0.1) in MCF-7 cells. The E2 induced mRNA expressions of PR (fold, 2.1±0.2) and of pS2 (fold, 1.5±0.1) was minimized by treating with FLA, although FLA alone did not affect these expressions (Fig. 3). The expression of G3PDH mRNA was measured as control. To reconfirm the mode of action of FLA on ERα, the binding activity was examined. As shown in Fig. 4A, FLA had neither the agonistic, nor antagonistic activity on ER in a yeast two-hybrid assay using rER. In the competitive binding assay using hER, FLA did not inhibit binding of ER (Fig. 4B). The results suggested that FLA of the basic structure had anti-estrogenic activity without binding to the ER receptor, though several derivatives of the flavone group have estrogenic activity (Innocenti et al., 2007; Hiremath et al., 2000).

AhR agonists induce rapid proteosome-dependent degradation of ER (Wormke et al., 2003). Furthermore, in breast cancer cells, ligand-bound AhR enhances ubiquitinated forms of ERα and proteasome-dependent degradation of ERα to repress the E2-induced transactivation (Wormke et al., 2000). As shown Figs. 1 and 2, FLA was suggested to be a ligand of AhR. Consequently, the protein level of ERα in the presence of FLA was determined in MCF-7 cells. FLA or E2 significantly decreased ERα protein level and FLA together with E2 enhanced this effect. Since TCDD, a ligand of AhR, activates proteasome-dependent degradation of ERα (Ohtake et al., 2003), the effect of protease inhibitor, a MG-132, was determined on ERα protein level. The results show that MG-132 prevented ERα from degradation by FLA (Fig. 5). Moreover, the results indicate that FLA induced the expression of CYP1A1 mRNA to enhance the degradation of ERα protein, and inhibited the expression of PR and pS2 mRNA through the AhR pathway.

It has been reported that some flavones show estrogenic activity through ER binding so that their intake has a preventive effect against prostate cancer (Raschke et al., 2006) and menopausal syndrome (Miller-Martini et al., 2001), and also has a stimulative effect on endometritis (Cline et al., 2004). Recently the Food Safety Commission of Japan published the upper-limit dose for soy isoflavone supplement to be 30 mg/day. Although Wood et al. (2006) reported that soy isoflavones had anti-estrogenic effects in the postmenopausal breast through ER signaling, we have presented in this work that FLA can be anti-estrogenic via AhR in MCF-7 cells. This means that other AhR agonists in food may potentially affect the action of estrogen.

Acknowledgments

We thank Dr. T. Matsuda (Kyoto University, Japan) for yeast transformed ARNT and AhR and Dr. H. Utsumi (Kyushu University, Japan) for providing chemicals. We appreciate Dr. S. Osada (Nagoya City University, Japan) for their valuable discussion and advice during this study, and also Dr.Y. K. Oh, living in Philadelphia, USA, for kind reviewing the English of this manuscript.

References

Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., Miller, C.A., Kato, T., Saeki, K., Matsuda, T., 2001. Indirubin and indigo are portent aryl hydrocarbon receptor ligands present in human urine. Journal of Biological Chemistry 276, 31475–31478.

Ahn, E., Akao, T., Nakamura, N., Komatsu, K., Nishihara, T., Hattori, M., 2004a. Screening of medicinal plant extracts for estrogenic activity in combination with a glycosidase treatment. Journal of Traditional Medicines 21, 81–86.

Ahn, E., Nakamura, M., Akao, T., Nishihara, T., Hattori, M., 2004b. Estrogenic and antiestrogenic activities of the roots of *Moghania philippinensis* and their constituents. Biological & Pharmaceutical Bulletin 27, 548–553.

Bingham, S.A., Atkinson, C., Liggins, J., Bluck, L., Coward, A., 1998. Phyto-estrogens; where are we now? British Journal of Nutrition 79, 393–406.

Brosens, J.J., Parker, M.G., 2003. Oestrogen receptor hijacked. Nature 423, 487–488.

Buchanan, D.L., Sato, T., Peterson, R.E., Cooke, P.S., 2000. Antiestrogenic effects of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin in mouse uterus: clinical role of the aryl hydrocarbon receptor in stromal tissue. Toxicological Sciences 57, 302–311.

Buchanan, D.L., Ohsako, S., Tohyama, C., Cooke, P.S., Iguchi, T., 2002. Dioxin inhibition of estrogen-induced mouse uterine epithelial mitogenesis involves change in cyclin and transforming growth factor-β-expression. Toxicological Sciences 66, 62–68.

Carver, L.C., Bradfield, C.A., 1997. Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophillin homolog in vivo. Journal of Biological Chemistry 272, 11452–11456.

Cline, J.M., Franke, A.A., Register, T.C., Golden, D.L., Adams, M.R., 2004. Effects of dietary isoflavone aglycones on the reproductive tract of male and female mice. Toxicologic Pathology 32, 91–99.

Denison, M.S., Whitlock Jr., J.P., 1995. Xenobiotic-inducible transcription of cytochrome P450 genes. Journal of Biological Chemistry 270, 18175–18178.

Diel, P., Schmidt, S., Vollmer, G., Janning, P., Upmeier, A., Michna, H., Bolt, H.M., Degen, G.H., 2004. Comparative response of three rat strains (DA/Han, Sprague–Dawley and Wistar) to treatment with environmental estrogens. Archive of Toxicology 78, 183–193.

Evans, R.M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240, 889–895.

Hamada, M., Satsu, H., Natsume, Y., Nishiumi, S., Fukuda, I., Ashida, H., Shimizu, M., 2006. TCDD-induced CYP1A1 expression, an index of dioxin toxicity, is suppressed by flavonoids permeating the human intestinal Caco-2 cell monolayers. Journal of Agricultural and Food Chemistry 54, 8891–8898.

Hiremath, S.P., Badami, S., Hunasagatta, S.K., Patil, S.B., 2000. Antifertility and hormonal properties of flavones of *Striga orobanchioides*. European Journal of Pharmacology 391, 193–197.

Hsieh, C.Y., Santell, R.C., Haslam, S.Z., Helferich, W.G., 1998. Estrogen effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. Cancer Research 58, 3833–3838.

Humfrey, C.D., 1988. Phytoestrogens and human health effects; weighing up the current evidence. Natural Toxins 6, 51–59.

Innocenti, G., Vegeto, E., Dall'Acqua, S., Ciana, P., Giorgetti, M., Agradi, E., Sozzi, A., Fico, G., Tome, F., 2007. In vitro estrogenic activity of *Achillea millefolium* L. Phytomedicine 14, 147–152.

Iwamoto, T., Nozawa, S., Yoshiike, M., Hoshino, T., Baba, K., Matsushita, T., Tanaka, S.N., Naka, M., Skakkebaek, N.E., Jorqensen, N., 2006. Semen quality of 324 fertile Japanese men. Human Reproduction 21, 760–765.

Jung, J., Ishida, K., Nishihara, T., 2004. Anti-estrogenic activity of fifty chemicals evaluated by in vitro assays. Life Sciences 74, 3065–3074.

Kim, J., Petz, L.N., Ziegler, Y.S., Wood, J.R., Potthoff, S.J., Nardulli, A.M., 2000. Regulation of the estrogen-responsive pS2 gene in MCF-7 human breast cancer cells. Journal of Steroid Biochemistry and Molecular Biology 74, 157–168.

Kuo, S.M., 2002. Flavonoids and gene expression in mammalian cells. Advances in Experimental Medicine and Biology 505, 191–200.

Kutzer, M.S., Xu, X., 1997. Dietary phytoestrogens. Annual Review of Nutrition 17, 353–381.

Lee, M.K., Yang, H., Ma, C.J., Kim, Y.C., 2007. Stimulatory activity of lignans from *Machilus thunbergii* on osteoblast differentiation. Biological & Pharmaceutical Bulletin 30, 814–817.

Mikamo, E., Harada, S., Nishikawa, J., Nishihara, T., 2003. Endocrine disruptors induce cytochrome P450 by affecting transcriptional regulation via pregnane X receptor. Toxicology and Applied Pharmacology 193, 66–72.

Miller, C.A., 1999. A human aryl hydrocarbon receptor signaling pathway constructed in yeast displays additive responses to ligand mixtures. Toxicology and Applied Pharmacology 160, 297–303.

Miller-Martini, D.M., Chan, R.Y., Ip, N.Y., Sheu, S.J., Wong, Y.H., 2001. A reporter gene assay for the detection of phytoestrogens in traditional Chinese medicine. Phytotherapy Research 15, 487–492.

Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S., Ustumi, H., 2000. Estrogenic activities of 517 chemicals by yeast two-hybrid assay. Journal of Health Science 46, 282–298.

Nishikawa, Y., 2003. Kankyo-Horumon. Nihon Hyoron-sha, Japan, p. 178.

Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y., Kato, S., 2003. Modulation of oestrogen receptor signaling by association with the activated dioxin receptor. Nature 423, 545–550.

Okamoto, Y., Suzuki, A., Ueda, K., Ito, C., Itoigawa, M., Furukawa, H., Nishihara, T., Kojima, N., 2006. Anti-estrogenic activity of prenylated isoflavones from millettia pachycarpa: implication of for pahrmacophores and unique mechanisms. Journal of Health Science 52, 1–6.

Petz, L.N., Ziegler, Y.S., Loven, M.A., Nardulli, A.M., 2002. Estrogen receptor α and activating protein-1 mediate estrogen receptor gene in MCF-7 breast cancer cells. Endocrinology 143, 4583–4591.

Raschke, M., Rowland, I.R., Magee, P.J., Pool-Zobel, B.L., 2006. Genistein protects prostate cells against hydrogen peroxide-induced DNA damage and induced expression of genes involved in the defence against oxidative stress. Carcinogenesis 27, 2322–2330.

Seo, H.S., Journe, F., Larsimont, D., Sotiriou, C., Leclercq, G., 2003. Decrease of estrogen receptor expression and associated ERE-dependent transcription in MCF-7 breast cancer cells after oligomycin treatment. Steroids 68, 257–269.

Speroff, L., 2000. A clinical understanding of the estrogen receptor. Annals of the New York Academy of Sciences 900, 26–39.

Stacey, M.R., Toscano, D.G., Mattingly, C.J., Toscano Jr., W.A., 1999. Estrogen receptor reduces CYP1A1 induction in cultured human endometrial cells. Journal of Biological Chemistry 274, 3430–3438.

Whitlock Jr., J.P., Okino, S.T., Dong, L., Ko, H.P., Clarke-katzenberg, R., Ma, Q., Li, H., 1996. Cytochromes P450 5: induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription. FASEB Journal 10, 809–818.

Wood, C.E., Resigter, T.C., Franke, A.A., Anthony, M.S., Cline, J.M., 2006. Dietary soy isoflavones inhibit estrogen effects in the postmenopausal breast. Cancer Research 66, 1241–1249.

Wormke, M., Stoner, M., Saville, B., Safe, S., 2000. Crosstalk between estrogen receptor α and the aryl hydrocarbon receptor in breast cancer cells involves unidirectional activation of proteasomes. FEBS Letters 478, 109–112.

Wormke, M., Stoner, M., Saville, B., Walker, K., Abdelrahim, M., Burghardt, R., Safe, S., 2003. The aryl hydrocarbon receptor mediated degradation of estrogen receptor α through activation of proteasomes. Molecular and Cellular Biology 23, 1843–1855.

Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Letters 418 (2007) 236–241
Neuroscience Letters
www.elsevier.com/locate/neulet

Memory impairment associated with a dysfunction of the hippocampal cholinergic system induced by prenatal and neonatal exposures to bisphenol-A

Kazuya Miyagawa, Minoru Narita *, Michiko Narita, Hisahiko Akama, Tsutomu Suzuki *

Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2-4-41 Ebara, Shinagawaku, Tokyo 142-8501, Japan

Received 2 November 2006; received in revised form 15 January 2007; accepted 28 January 2007

Abstract

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4′-isopronylidenediphenol, called bisphenol-A. In the previous study, we reported that exposure to bisphenol-A induced the abnormality of dopamine receptor functions in the mouse limbic area, resulting in a supersensitivity of drugs of abuse-induced pharmacological actions. The present study was undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnormalities such as anxiogenic behavior, motor learning behavior, or memory. In the present study, adult female mice were chronically treated with bisphenol-A-admixed powder food from mating to weaning. All experiments were performed using male pups. Here we found that prenatal and neonatal exposures to bisphenol-A failed to induce anxiogenic effects and motor-learning impairment using the light-dark test, elevated plus maze test, and rota-rod test. On the other hand, we found that prenatal and neonatal exposures to bisphenol-A induced the memory impairment using the step-through passive avoidance test. Immunohistochemical study showed the dramatic reduction in choline acetyltransferase-like immunoreactivity, which is a marker of acetylcholine (ACh) production, in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A. These results suggest that chronic exposures to bisphenol-A could induce the memory impairment associated with the reduction in ACh production in the hippocampus.

Keywords: Bisphenol-A; Memory; Choline acetyltransferase; Hippocampus; Endocrine disrupting chemicals

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4′-isopronylidenediphenol, called bisphenol-A, which is used in the manufacture of many types of products.

Our recent studies suggest that exposure to bisphenol-A during prenatal and postnatal development has long-lasting effects on central dopaminergic systems linked to rewarding effects, as well as drug addiction induced by drugs of abuse [13–16,21,22]. The fetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Although bisphenol-A, has weak estrogenic activity, prenatal and neonatal exposures to 17β-estradiol failed to induce the supersensitivity of morphine [13]. Furthermore, although it is well known that bisphenol-A disrupts thyroid hormone, prenatal and neonatal exposures to propylthiouracil, an inhibitor of thyroid hormone synthesis, reduced the activation of dopaminergic neurons (unpublished observation). These findings indicate that the disruption of dopaminergic neuron development induced by prenatal and neonatal exposures to bisphenol-A can be mediated by nonhormonergic actions of bisphenol-A. Furthermore, our preliminary biochemical studies showed that bisphenol-A has an affinity for nonspecific binding sites. Thus since its action site remained unclear, it is most likely that prenatal and neonatal exposures to bisphenol-A induce other behavioral abnormalities associated with the alternation not only of the dopaminergic system, but also of other neurotransmissions. The present study was then undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnor-

* Corresponding authors. Tel.: +81 3 5498 5627; fax: +81 3 5498 5627.
E-mail addresses: narita@hoshi.ac.jp (M. Narita), suzuki@hoshi.ac.jp (T. Suzuki).

0304-3940/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2007.01.088

malities such as anxiogenic behavior, motor learning behavior, and memory.

The low-dose endocrine disrupting actions of various environmental toxicants are serious problems. However, little is known about the effects on the central nervous system induced by low-dose bisphenol-A. We also investigated whether prenatal and neonatal exposures to low-dose bisphenol-A in mice could induce behavioral abnormalities.

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

All experiments were performed using 7–11-week-old male C57BL/6J mice (Japan SLC Inc., Shizuoka, Japan) that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd., Osaka, Japan). Adult female mice (10 weeks old) were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 30 ng/g diet (Low), and 2 mg/g diet (High) from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, the animals did not show any body weight loss. In the present study, at least 10 dams were used per group. We randomly selected a few pups per litter and housed to undergo the behavioral tests. To obtain unbiased results, we appropriately distributed mice for each behavioral study.

To investigate changes in anxiogenic-like effects, the mice were tested using the light–dark paradigm [2,19]. We used a box consisting of a small (18 cm × 13 cm × 18 cm) dimly lit compartment with black walls and a black floor, connected by a small opening (5 cm × 5 cm) to a large (18 cm × 18 cm × 18 cm) intensely lit (500 lux) compartment with white walls and a white floor. Each animal (n = 11–14/group, 7 weeks old) was placed in the dark compartment at the beginning of the observation session. The compartment entry and exit were defined as all four paws into and out of the chamber, respectively. The time spent in the lit compartment was recorded for 10 min. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

The elevated plus-maze consists of two opposing open arms (30 cm × 6 cm × 0.3 cm) and two opposing enclosed arms (30 cm × 6 cm × 15 cm) that are connected by a central platform (9 cm × 9 cm, 100 lux), thus forming the shape of a plus sign. Each animal (n = 5–7/group, 7 weeks old) was tested using the elevated plus-maze in each experiment. We recorded for 5 min the time spent in open or enclosed arms and the time spent entering into the arms. The results were calculated as mean ratios of the time spent in the open arms to the total time spent in both the open and enclosed arms. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

To investigate possible changes in motor learning impairment with prenatal and neonatal exposures to bisphenol-A, the mice were tested using the rota-rod performance procedure (rota-rod test). The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a nonslippery surface. The 30-cm-long rod was placed at a height of 15 cm from the base and was divided into five equal sections by six disks. Thus the mice were tested simultaneously on the apparatus with a rod-rotating speed of 10 rpm. Each animal (n = 6–7/group, 9 weeks old) was placed on the rotating rod, requiring forward locomotion. Each animal was tested for a total of 5 min. If/when a mouse fell from the rod it was immediately replaced. We measured the time until falling the first time and the numbers of falls during the 5 min as the indicators of motor impairment twice a day. The score was the mean of latencies or numbers of falls in two trials. The data are presented as the mean with S.E.M. Statistical analyses were performed using two-way ANOVA with Bonferroni/Dunnett's test.

The experimental apparatus for the step-through passive avoidance test is a shuttle box that is divided into an illuminated small compartment (12 cm × 5 cm × 14 cm) and a dark/large compartment (25 cm × 25 cm × 20 cm) by a wall with a guillotine door. On the first day (conditioning day), each animal (n = 5–7/group, 11 weeks old) was placed in the illuminated compartment. After 90 s, the door was opened and the mouse was freely moved into the dark compartment. The door was closed as soon as the mouse stepped into the dark compartment, and an inescapable foot shock (0.5 mA, 0.5 s) was delivered through the grid floor. After 48 h from conditioning day, a retention test was started. The retention test was performed in a similar manner but without the electric shock and the step-through latency to the dark compartment was recorded. The maximal cut-off time for step-through latency was 30 min. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way or two-way ANOVA with Bonferroni/Dunnett's test.

In the immunohistochemical approach, the mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion fixed with 4% paraformaldehyde (pH 7.4). The brains were then quickly removed after perfusion, and thick coronal sections of the midbrain, including the hippocampus, were initially dissected using Brain Blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 h. After the brains were permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −30 °C until use. Frozen 8-μm-thick coronal sections were cut with a cryostat (CM1510; Leica, Heidelberg, Germany) and thaw-mounted on poly-L-lysine-coated glass slides. The brain sections were blocked in 3% normal horse serum with 0.2% triton (for choline acetyltransferase (ChAT); Chemicon International Inc., CA, USA) and 10% normal goat serum (NGS) (for NeuN; Chemicon) in 0.01 M PBS for 1 h at room temperature. The primary antibody of ChAT (1:100) or NeuN (1:250) was diluted in 0.01 M PBS containing 3% NHS with 0.2% triton or 10% NGS and incubated for 2 days at 4 °C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 or Alexa 546 for 2 h at room temperature. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA). Fluorescence immunolabeling was detected using

a light microscope (AX-70; Olympus Optical, Tokyo, Japan) and photographed with a digital camera (Polaroid PDMCII/OL; Olympus Optical). Digitized images of the dentate gyrus were captured at a resolution of 140–200 pixels with a digital camera (Polaroid PDMCII/OL; Olympus). The density of ChAT labeling was measured with a computer-assisted imaging analysis system (Image J program, developed at the National Institutes of Health available at http://rsb.info.nih.gov/ij). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity; this provided an image with immunoreactive material appearing in black pixels and non-immunoreactive material in white pixels. A standardized rectangle was positioned over the hippocampus of control mice. The area and density of pixels within the threshold value representing immunoreactivity were calculated and the integrated density was the product of the area and density. The same box was then "dragged" to the corresponding position on the hippocampus of bisphenol-A treated mice, and the integrated density of pixels within the same threshold was again calculated. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

In the present study, prenatal and neonatal exposures to bisphenol-A failed to induce anxiogenic-like effects using the light–dark paradigm (Fig. 1A). As another measurement of anxiety, the mice prenatally and neonatally exposed to bisphenol-A were evaluated by the elevated plus-maze paradigm. The percentage of time spent in the open arms in the mice prenatally and neonatally exposed to bisphenol-A are shown in Fig. 1B. The prenatal and neonatal exposures to bisphenol-A failed to affect the percentage of time spent in the open arms. These results suggest that prenatal and neonatal exposures to bisphenol-A induced no anxiogenic-like behaviors. In the light–dark paradigm, the time spent in the light compartment of the mice prenatally and neonatally exposed to low-dose of bisphenol-A was increased as compared with the mice prenatally and neonatally exposed to high-dose of bisphenol-A (Fig. 1A). Although it is very difficult to explain the difference between the effect of low and high doses of bisphenol-A, it is necessary to consider the effects of the difference of the dosage on the difference of the action.

To investigate possible changes in motor learning impairment with prenatal and neonatal exposures to bisphenol-A, the mice were tested using the rota-rod test. In the present study, the mice prenatally and neonatally exposed to low or high doses of bisphenol-A progressively improved their skill in the rota-rod test, as the control mice did (Fig. 1C and D). The improvement of latency to fall and the number of fallings in the rota-rod test were indistinguishable between control and bisphenol-A treated mice (Fig. 1C and D). These results suggest that prenatal and neonatal exposures to bisphenol-A have no direct effect on motor skill learning.

The influence of prenatal and neonatal exposures to bisphenol-A on memory processes in mice was evaluated by the step-through passive avoidance test. In the conditioning trial, the step-through latency of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A was similar to that of control mice (Fig. 1E). Although the latency to step-through increased in all groups compared to the latency shown at conditioning, the latencies to step into the dark compartment dramatically decreased in mice prenatally and neonatally exposed to bisphenol-A as compared to control mice ($F_{(2,30)} = 5.766$, $p < 0.05$, Fig. 1F). These results strongly suggest that chronic treatment with low and high doses of bisphenol-A induced the memory impairment. The contextual fear conditioning is hippocampal-dependent memory. Therefore, we next investigated the morphological and/or functional changes in the hippocampus of mice prenatally and neonatally exposed to low and high doses of bisphenol-A.

Immunohistochemical study showed that prenatal and neonatal exposures to low and high doses of bisphenol-A dramatically decreased the level of choline acetyltransferase-like immunoreactivity (ChAT-IR) in the widespread regions of the hippocampus compared to control (Fig. 2Ai–iii). Especially as shown in high magnification images, cholinergic fiber was dramatically decreased in mice prenatally and neonatally exposed to low and high doses of bisphenol-A compared to control (Fig. 2Bi–iii). Furthermore, these phenomena were observed in several regions of the hippocampus, such as CA1, CA2, and CA3 (data not shown). Using semi-quantitative analysis, prenatal and neonatal low and high doses of bisphenol-A produced a significant decrease in the level of ChAT-IR in the hippocampus (Low: $64.3 \pm 5.6\%$ of control mean, (***) $p < 0.001$ versus control mice; High: $50.0 \pm 3.8\%$ of control mean, (***) $p < 0.001$ versus control mice, Fig. 2C). It is widely accepted that cholinergic function in the hippocampus is important for the learning and memory processes [1,4,12]. Among the cholinergic parameters described for the brains of Alzheimer's disease patients, the decrease in ChAT activity is the most prominent and provides an excellent biochemical correlation to the severity of dementia. These reports strongly support our findings that the memory impairment corresponded well to the dysfunction of cholinergic neuron in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A. Prenatal and neonatal exposure to bisphenol-A failed to affect the NeuN-IR in the dentate gyrus (Fig. 2Di–iii) and other regions of hippocampus compared to control, indicating that prenatal and neonatal exposure to bisphenol-A failed to cause cell death or layer formation on the mature pyramidal or granular cells in the hippocampus. Taken together, we hypothesized that although the prenatal and neonatal exposure to bisphenol-A could induce the dynamic morphological changes in cholinergic fibers associated with the disruption of the neuron development, it did not modify the hippocampal content of all immunogens.

It is widely accepted that one of the most common endocrine disruptors, bisphenol-A, has weak estrogenic activity. Moreover, endogenous estrogen, 17β-estradiol, plays the critical role in the neurotransmission of the hippocampus associated with spinogenesis or neuroprotection [5,18]. Furthermore, many cholinergic neurons also express the growth-associated protein GAP-43, may be taken as a marker of neurite outgrowth [11]. Estrogens, among other factors, up-regulate the expression of GAP-43 in the developing and adult brain [9]. On the other hand, bisphenol-A also disrupts thyroid hormone. Thyroid hormone deficiency during brain development disrupts on the activities

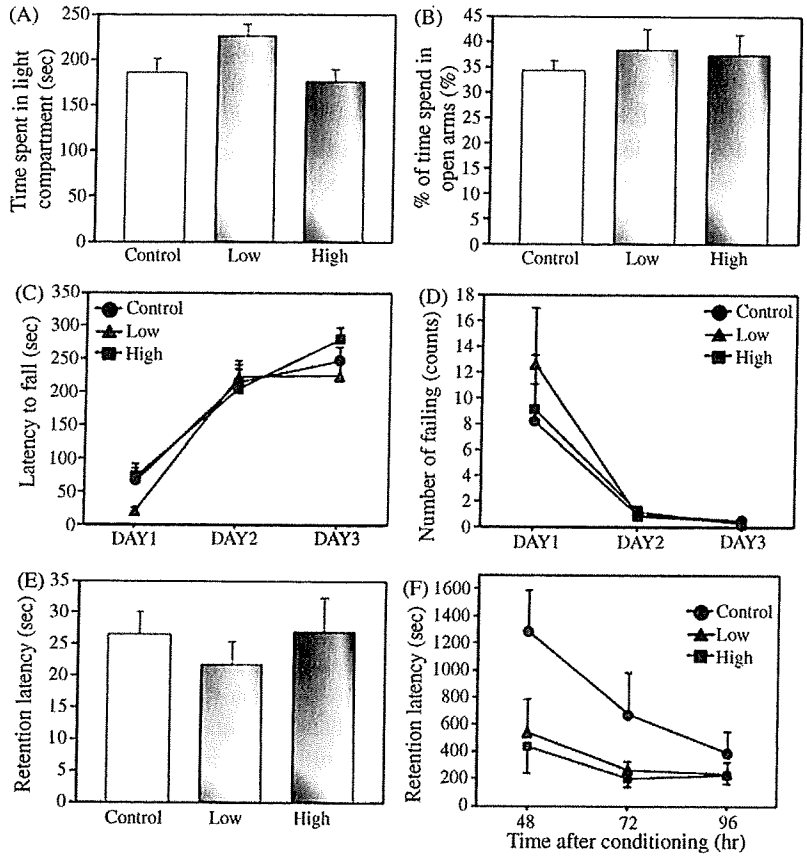


Fig. 1. Behavioral analysis in adult mice prenatally and neonatally exposed to bisphenol-A. (A) Lack of anxiogenic effects by prenatal and neonatal exposures to bisphenol-A using the light–dark test procedure. Prenatal and neonatal exposures to bisphenol-A failed to change the time spent in the lighted compartment as compared with control mice. On the other hand, time spent in the light compartment of the mice prenatally and neonatally exposed to low-dose of bisphenol-A was increased as compared with the mice prenatally and neonatally exposed to high-dose of bisphenol-A (All group: $F_{(2,35)}=3.467$, $p<0.05$, Control vs. Low: $F_{(1,22)}=3.790$, $p>0.05$, Control vs. High: $F_{(1,25)}=0.227$, $p>0.05$, Low vs. High: $F_{(1,23)}=7.438$, $p<0.05$). Each column represents the mean with S.E.M. of 11–14 mice/group. (B) Lack of anxiogenic effect by prenatal and neonatal exposures to bisphenol-A using the elevated-plus-maze procedure. Prenatal and neonatal exposures to bisphenol-A failed to change the percentage of time spent in the open arms (All group: $F_{(2,15)}=0.391$, $p>0.05$, Control vs. Low: $F_{(1,9)}=1.128$, $p>0.05$, Control vs. High: $F_{(1,11)}=0.429$, $p>0.05$, Low vs. High: $F_{(1,10)}=0.044$, $p>0.05$). Each column represents the mean with SEM of 5–7 mice/group. (C and D) Lack of motor learning impairment by prenatal and neonatal exposures to bisphenol-A using the rota-lod test. (C) The time that the animal remained on a rotating rod at 10 rpm was measured twice a day. A maximum of 300 s was allowed for each animal per trial. The score was the mean of latencies in two trials (All group: $F_{(2,34)}=0.283$, $p>0.05$, Control vs. Low: $F_{(1,22)}=1.045$, $p>0.05$, Control vs. High: $F_{(1,24)}=0.284$, $p>0.05$, Low vs. High: $F_{(1,22)}=2.271$, $p>0.05$). Each point represents the mean with S.E.M. of 6–7 mice/group. (D) The number of falls from a rotating rod to the ground during 300 s was counted twice a day. The score was the mean of numbers of fallings in two trials (All group: $F_{(2,34)}=0.318$, $p>0.05$, Control vs. Low: $F_{(1,22)}=0.645$, $p>0.05$, Control vs. High: $F_{(1,24)}=0.027$, $p>0.05$, Low vs. High: $F_{(1,22)}=0.259$, $p>0.05$). Each point represents the mean with S.E.M. of 6–7 mice/group. (E and F) The effects of prenatal and neonatal exposures to bisphenol-A on performance in a step-through passive avoidance procedure. (E) At conditioning, the mice were placed in the lighted compartment of a two-compartment box and received a foot shock as soon as they stepped into the dark compartment. The step-through latency of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A was similar to that of the control mice (All group: $F_{(2,15)}=0.401$, $p>0.05$, Control vs. Low: $F_{(1,9)}=0.882$, $p>0.05$, Control vs. High: $F_{(1,10)}=0.003$, $p>0.05$, Low vs. High: $F_{(1,11)}=0.584$, $p>0.05$). (F) Prenatal and neonatal exposures to low and high doses of bisphenol-A induced a significant memory impairment (All group: $F_{(2,30)}=5.766$, $p<0.05$, Control vs. Low: $F_{(1,18)}=6.246$ $p<0.05$, Control vs. High: $F_{(1,20)}=9.167$, $p<0.05$, Low vs. High: $F_{(1,22)}=0.222$, $p>0.05$). Each point represents the mean with S.E.M. of 5–7 mice/group.

of enzymes of central acetylcholine metabolism, the activities of ChAT and acetylcholinesterase in the hippocampus [8]. Actually, exposure to polychlorinated biphenyls, well known as one of the most common thyroid hormone disruptors, suppressed ChAT activity and spatial learning and memory deficits [3,6]. According to these reports, we hypothesized that the memory impairment induced by prenatal and neonatal exposures to bisphenol-A could be mediated by the disruption of endogenous hormones in the developing brain.

In the present study, we found that memory impairment associated with a drastic reduction of ChAT-IR in the hippocampus was induced by prenatal and neonatal exposures not only to high-dose, but also to low-dose bisphenol-A. Although it is very difficult to explain where the primary site of bisphenol-A is, we therefore must take into account its mechanism through nonhormonergic effects.

Knaak and Sullivan first reported the metabolic fate of bisphenol-A in rats, showing that the major metabolite in urine was the glucuronide of bisphenol-A; considerable amounts of free bisphenol-A and hydroxylated bisphenol-A were found in feces [7]. Many reports have shown that bisphenol-A is metabolized and excreted rapidly [10,17,20]. Taken together, it is almost impossible that the bisphenol-A remaining in the adult brain of mice directly affects CNS. Therefore, prenatal and neonatal

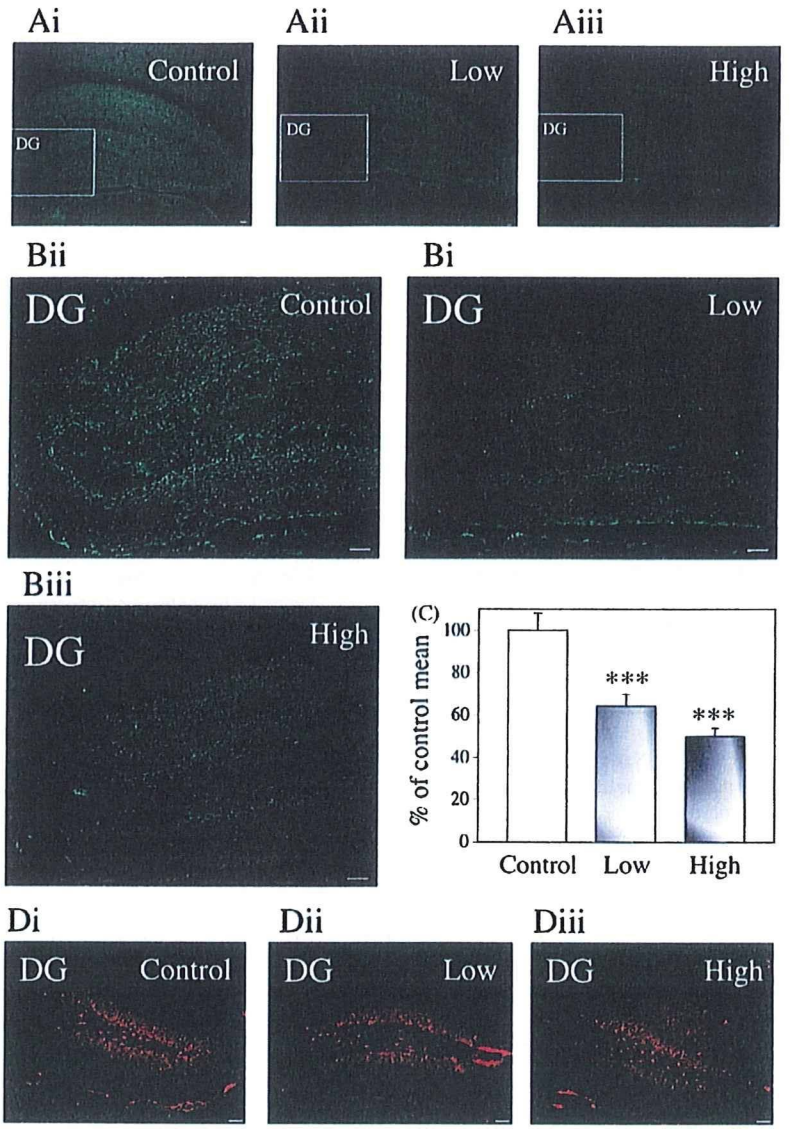


Fig. 2. Dramatic reduction in ChAT-like immunoreactivity in the hippocampus of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A. (Ai–iii) Prenatal and neonatal exposures to low (Aii) and high doses (Aiii) of bisphenol-A dramatically decreased the level of ChAT-IR in the hippocampus compared to control (Ai). (Bi–iii) High magnification images showed that ChAT-IR in the DG. ChAT-IR was dramatically decreased in the DG by prenatal and neonatal exposures to low (Bii) and high doses (Biii) as compared to control (Bi). (C) A semi-quantitative analysis of ChAT-IR was performed using Image J (Low: 64.3 ± 5.6% of control mean, (***) $p < 0.001$ vs. control mice; High: 50.0 ± 3.8% of control mean, (***) $p < 0.001$ vs. Control mice). (Di–iii) On the other hand, prenatal and neonatal exposure to low (Dii) and high doses (Diii) of bisphenol-A faild to change NeuN-IR in the DG compared to control (Di). Each column represents the mean ± S.E.M. of three independent samples. DG: dentate gyrus. Scale bars: 50 μM.

exposures to bisphenol-A disrupt the neuron development, resulting in behavior abnormalities in the adult animals.

In conclusion, the present findings provide direct evidence that prenatal and neonatal exposures not only to high-dose, but also to low-dose of bisphenol-A dramatically decreases the cholinergic transmission in the adult brain, resulting in learning and memory deficits.

Acknowledgements

This work was supported in part by grants from the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology of Japan. We wish to thank Mr. Keiichi Niikura, Miss. Kana Nanjo and Miss. Sayaka Enomoto for their expert technical assistances of immunohistochemistry.

References

[1] R.T. Bartus, R.L. Dean 3rd, B. Beer, A.S. Lippa, The cholinergic hypothesis of geriatric memory dysfunction, Science 217 (1982) 408–414.
[2] A. Bilkei-Gorzo, I. Gyertyan, G. Levay, mCPP-induced anxiety in the light-dark box in rats–a new method for screening anxiolytic activity, Psychopharmacology (Berl) 136 (1998) 291–298.
[3] D.A. Donahue, E.J. Dougherty, L.A. Meserve, Influence of a combination of two tetrachlorobiphenyl congeners (PCB 47; PCB 77) on thyroid status, choline acetyltransferase (ChAT) activity, and short- and long-term memory in 30-day-old Sprague-Dawley rats, Toxicology 203 (2004) 99–107.

[4] P. Dutar, M.H. Bassant, M.C. Senut, Y. Lamour, The septohippocampal pathway: structure and function of a central cholinergic system, Physiol. Rev. 75 (1995) 393–427.
[5] E. Gould, C.S. Woolley, M. Frankfurt, B.S. McEwen, Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood, J. Neurosci. 10 (1990) 1286–1291.
[6] L.M. Juarez de Ku, M. Sharma-Stokkermans, L.A. Meserve, Thyroxine normalizes polychlorinated biphenyl (PCB) dose-related depression of choline acetyltransferase (ChAT) activity in hippocampus and basal forebrain of 15-day-old rats, Toxicology 94 (1994) 19–30.
[7] J.B. Knaak, L.J. Sullivan, Metabolism of bisphenol A in the rat, Toxicol. Appl. Pharmacol. 8 (1966) 175–184.
[8] Z. Li, R. Yang, Z. Chen, Effects of iodine and thyroid hormone deficiency during brain development on activities of cholinergic neurone-related enzymes in central nervous system of rats, Zhonghua Yu Fang Yi Xue Za Zhi 30 (1996) 337–339.
[9] R.H. Lustig, M. Sudol, D.W. Pfaff, H.J. Federoff, Estrogenic regulation and sex dimorphism of growth-associated protein 43 kDa (GAP-43) messenger RNA in the rat, Brain Res. Mol. Brain Res. 11 (1991) 125–132.
[10] J.B. Matthews, K. Twomey, T.R. Zacharewski, In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta, Chem. Res. Toxicol. 14 (2001) 149–157.
[11] M. McKinney, C. Kent, Differential expression of GAP-43 mRNA in adult central cholinergic neuronal populations, Brain Res. Mol. Brain Res. 23 (1994) 213–220.
[12] M. Miyamoto, J. Kato, S. Narumi, A. Nagaoka, Characteristics of memory impairment following lesioning of the basal forebrain and medial septal nucleus in rats, Brain Res. 419 (1987) 19–31.
[13] M. Miyatake, K. Miyagawa, K. Mizuo, M. Narita, T. Suzuki, Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol-A in neurones and astrocytes, J. Neuroendocrinol. 18 (2006) 434–444.
[14] K. Mizuo, M. Narita, K. Miyagawa, E. Okuno, T. Suzuki, Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice, Neurosci. Lett. 356 (2004) 95–98.
[15] K. Mizuo, M. Narita, T. Yoshida, T. Suzuki, Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice, Addict. Biol. 9 (2004) 19–25.
[16] M. Narita, K. Miyagawa, K. Mizuo, T. Yoshida, T. Suzuki, Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect, Neurosci. Lett. 402 (2006) 249–252.
[17] L.H. Pottenger, J.Y. Domoradzki, D.A. Markham, S.C. Hansen, S.Z. Cagen, J.M. Waechter Jr., The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration, Toxicol. Sci. 54 (2000) 3–18.
[18] J. Prange-Kiel, G.M. Rune, Direct and indirect effects of estrogen on rat hippocampus, Neuroscience 138 (2006) 765–772.
[19] N.L. Schramm, M.P. McDonald, L.E. Limbird, The alpha(2a)-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety, J. Neurosci. 21 (2001) 4875–4882.
[20] R.W. Snyder, S.C. Maness, K.W. Gaido, F. Welsch, S.C. Sumner, T.R. Fennell, Metabolism and disposition of bisphenol A in female rats, Toxicol. Appl. Pharmacol. 168 (2000) 225–234.
[21] T. Suzuki, K. Mizuo, K. Miyagawa, M. Narita, Exposure to bisphenol-A affects the rewarding system in mice, Nihon Shinkei Seishin Yakurigaku Zasshi 25 (2005) 125–128.
[22] T. Suzuki, K. Mizuo, H. Nakazawa, Y. Funae, S. Fushiki, S. Fukushima, T. Shirai, M. Narita, Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptor-mediated action in mice: enhancement of the methamphetamine-induced abuse state, Neuroscience 117 (2003) 639–644.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 364 (2007) 276–282
BBRC
www.elsevier.com/locate/ybbrc

Molecular mechanism of transcriptional repression of AhR repressor involving ANKRA2, HDAC4, and HDAC5

Motohiko Oshima [a,b], Junsei Mimura [a,b], Masayuki Yamamoto [a], Yoshiaki Fujii-Kuriyama [a,b,*]

[a] *Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan*
[b] *SORST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi 332-0012, Japan*

Received 14 September 2007
Available online 11 October 2007

Abstract

The Aryl hydrocarbon receptor repressor (AhRR) has been proposed to inhibit Aryl hydrocarbon receptor (AhR) activity by competing with AhR for forming a heterodimer with AhR nuclear translocator (Arnt) and subsequently binding to the xenobiotic responsive elements (XRE). However, the precise mechanism of AhRR inhibitory activity remains unknown. Analysis of the inhibitory activity of AhRR on the expression of a TK promoter-driven reporter has localized a core repressor domain in the sequence of amino acid residue 555–701. The inhibitory activity of AhRR is sensitive to a histone deacetylase (HDAC) inhibitor, trichostatin A. By using the yeast two-hybrid screening method with the C-terminal sequence of AhRR as bait, we identified a binding partner, Ankyrin-repeat protein2 (ANKRA2), a protein known to interact with HDAC4 and HDAC5. RNA interference experiments using ANKRA2 and AhRR siRNAs indicate that ANKRA2 is important for transcriptional repression by AhRR. We have found that under normal conditions, *CYP1A1* gene is kept silent in MEF cells by AhRR/Arnt heterodimer, which binds to the XRE sequence in its promoter and recruits ANKRA2, HDAC4, and HDAC5 as co-repressors.

Keywords: Aryl hydrocarbon receptor (AhR); AhR repressor (AhRR); ANKRA2; HDAC4; HDAC5; CYP1A1; RFXANK

The Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to a superfamily with basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) structural motifs and functions as an intracellular mediator of xenobiotic signaling pathways [1]. Normally, AhR exists within the cytoplasm in association with a complex of HSP90, XAP2, and p23. Upon binding a ligand such as tetrachlorodibenzo-*p*-dioxin (TCDD), the AhR complex translocates into the nucleus and forms a heterodimer with the structurally related AhR nuclear translocator (Arnt) [1]. Thereupon, the AhR/Arnt heterodimer binds to XRE (xenobiotic responsive elements) sequences in the promoter regions of the target genes encoding drug-metabolizing enzymes, such as *CYP1A1* and *CYP1B1* to enhance their expressions [2]. The AhR signaling pathway mediates not only the adaptive response required for the detoxification of xenobiotics, but also a variety of xenobiotics-induced toxicological effects such as tumor promotion, teratogenesis, and endocrine disruption [3–7]. In addition, AhR is also known to mediate multiple physiologic processes such as female reproduction [8,9].

During the course of our study on the AhR transcription factor, we found a novel bHLH-PAS family protein with a high similarity to AhR in the N-terminal bHLH-PAS A domain. In contrast, its C-terminal region shares a minimal degree of similarity to that of AhR and lacks the obvious PAS B domain of the ligand-binding site in AhR [10]. In transient DNA transfection assays, we found

* Corresponding author. Address: Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan. Fax: +81 29 853 7318.
E-mail address: ykfujii@tara.tsukuba.ac.jp (Y. Fujii-Kuriyama).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.09.131

that this novel protein inhibits AhR-dependent transactivation of the XRE-driven gene, and thus designated it AhR repressor (AhRR) [10].

Recently, AhRR orthologs have been reported in human [11,12], rat [11,13], and fish [14,15] and their genes have three conserved XRE sequences in the 5′-flanking promoter region. Accordingly, it has been reported that the AhRR expression is activated by the AhR/Arnt heterodimer in some cell lines [11,12] and multiple tissues of various species [10,12–14], indicating that AhRR participates in a negative feedback loop in the AhR signaling pathway [10,14,15].

Although the precise mechanism of inhibitory activity of AhRR remains to be elucidated, it has been proposed that AhRR competes with AhR for heterodimerization with Arnt and binding to the XRE sequence, a property that is likely to be mediated by N-terminal bHLH-PAS domains of these proteins [10].

In this study, we report that AhRR has a transcriptional repression domain within its C-terminal region, which exhibits a trichostatin A (TSA)-sensitive HDAC activity. By Cytotrap yeast two-hybrid screening with the C-terminal sequence of AhRR used as bait, we isolated Ankyrin-repeat protein2 (ANKRA2) as a binding partner to the AhRR C-terminal sequence.

Materials and methods

Plasmids. Fragments for AhRR(1–701 a.a.), AhRR(1–342 a.a.), AhRR(342–701 a.a.), AhRR(342–478 a.a.), AhRR(478–701 a.a.), AhRR(478–555 a.a.), AhRR(555–701 a.a.) were excised from pBOS-AhRR [10] and cloned into the pBOSGAL4DBD vector [16]. pG3TK-Luc was produced by inserting three copies of the GAL4 binding site excised from pG5EC and TK promoter sequence excised from pBSCAT2 into the XhoI site of pGL3 vector (Clontech). pBOSHA-AhRR was constructed as follows: pBOST7HA vector was constructed by inserting the blunt-ended BglII/PstI fragment of pGADT7 vector containing T7 promoter and HA epitope Tag into blunt-ended BamHI site of pEFBOS vector [17]. The EcoRI/SalI fragment excised from pBSKAhRR (Mimura, unpublished data) was inserted into the EcoRI and SalI site of pBOST7HA. pBOS-FLAG-ANKRA2 was constructed as follows: pBOST7FLAG vector was constructed by inserting the fragment containing the T7 promoter and FLAG epitope Tag cleaved from pGADT7FLAG vector into blunt-ended BamHI site of pEFBOS vector. Mouse ANKRA2 cDNA was amplified with a pair of primers, 5′-CatcgatACATGGCTACATCTGCAAAT-3′ and 5′-CggatccTCACTCCCTGATGTTCTGAA-3′ as the 5′ and 3′ primers, respectively. The amplified cDNA fragment was digested with ClaI and BamHI, and inserted into the ClaI and BamHI site of pBOST7FLAG. Expression plasmids encoding HDAC4-FLAG and HDAC5-FLAG were kindly provided by Dr. Stuart L. Schreiber (Harvard University, MA, USA).

Antibody production. Recombinant glutathione S-transferase (GST)-tagged mouse AhRR (342–701 a.a.) and recombinant maltose-binding protein (MBP)-tagged mouse AhRR (342–701 a.a.) were expressed in *Escherichia coli* and purified with Glutathione Sepharose 4B (Amersham Biosciences) and amylose resin (New England Biolabs), respectively, according to the manufacturer's protocols. Polyclonal rabbit antisera were raised against the recombinant GST-AhRR (342–701 a.a.) and further affinity-purified with the recombinant MBP-AhRR (342–701 a.a.) (Hokudo Inc., Japan).

Cell culture. Mouse embryonic fibroblast (MEF) cells were isolated from C57B/6J mice. COS-7, MEF, and Hepa-1c1c7 (Hepa-1) cells were maintained in high glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Invitrogen) under 5.0% CO_2 at 37 °C.

Luciferase assay. Hepa-1 cells (5.0×10^4 cells/well) were grown in 24-well dishes for 24 h and were transfected with the expression plasmids indicated in the figure legends, pG3TK-Luc and the expression plasmids for sea pansy luciferase as an internal control using Lipofectamine™ (Invitrogen). Forty-eight hours after transfection, the cells were harvested and luciferase was quantified by using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocols. For control of transfection efficiency, firefly luciferase activity was normalized to cotransfected sea pansy luciferase activity as a standard.

Coimmunoprecipitation and immunoblot analysis. Cell lysates from the transfected COS-7 and MEF cells were prepared as described and used for immunoblot analysis either directly or after immunoprecipitation. Immunoprecipitation with anti-Flag M2 agarose (Sigma) or anti-HA agarose (Sigma) was performed for 12 h and the immunoprecipitates were washed according to the published procedure for immunoblot analysis. Immunoblot analysis was performed as described [7] using anti-FLAG (Sigma), anti-HA (Sigma), anti-Tubulin (Sigma), and anti AhRR antibodies.

RNA interference experiments. The siRNAs for mouse ANKRA2 or mouse AhRR were designed and synthesized by B-Bridge International Inc. The coding sequences were: ANKRA2, (5′-AGGAAAAGGUCGAG AAAGUdTdT-3′) and AhRR, (5′-GGAAAGGCCUUGUGGCUAAdT dT-3′). Hepa-1 cells or MEF cells (5.0×10^4 cells/well) were transfected with siRNA for ANKRA2 (20 pmol) or AhRR (50 pmol) with or without expression plasmids by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Quantification of mRNA. Total RNAs are extracted from cells using Isogen (Nippon Gene, Tokyo) and reverse transcribed by Superscript II (Invitrogen). Real-time PCR was carried out in ABI PRISM 7700 sequence detection system using the following primer sets: ANKRA2, forward (5′-TCTACCACACCTCTGTTAGC-3′) and reverse (5′-GCA CTTTCTCGACCTTTTCC-3′); AhRR, forward (5′-GCTTTCTGTCCT GCGCCTC-3′) and reverse (5′-TCCTTCCTGCACGGGGAAC-3′); CY P1A1, forward (5′-GGACATTTGAGAAGGGCCAC-3′) and reverse (5′-CGTCCAGCTTCCTGTCCTGA-3′); actin, forward (5′-GACAGGAT GCAGAAGGAGAT-3′) and reverse (5′-TTGCTGATCCACATCTGC TG-3′).

Cytotrap yeast two-hybrid assay. The CytoTrap™ (Stratagene) yeast screening was performed with a murine thymus cDNA library (Stratagene) and pSos-AhRR (342–701) as prey and bait, respectively, according to the manufacturer's instructions.

Results

Functional characterization of the AhRR transcriptional repression domain

We previously reported that AhRR inhibits the transcription activity of Arnt [10]. In order to confirm transcriptional repression activity and to localize the transcriptional repression domain of AhRR, we fused a series of AhRR deletion mutants to the GAL4 DNA binding domain (GAL) (Fig. 1A). These fragments were transfected into Hepa-1 cells, along with a luciferase reporter gene driven by three GAL4 binding sites and the TK promoter (Fig. 1A). The luciferase activity driven by the TK promoter was repressed 5.2-fold by the transfection with plasmid, GAL4DBD-AhRR (1–701) (Fig. 1B). Plasmids encoding the fusion proteins, GAL4DBD-AhRR (342–701), (478–701), and (555–701) also repressed luciferase expression 5.6-, 4.9- and 3.7-fold, respectively, while plasmids of AhRR (1–342), AhRR (342–478), and AhRR

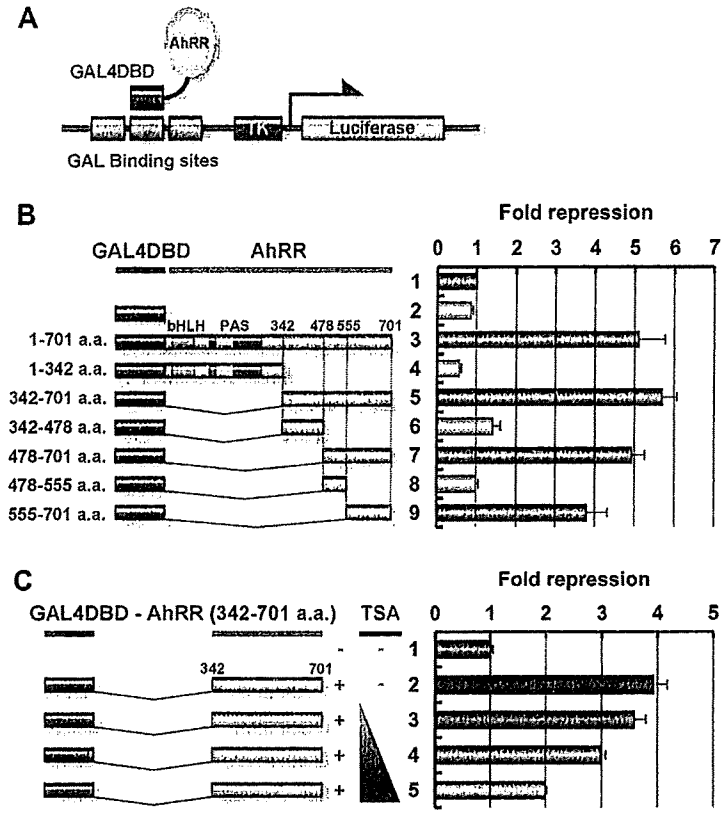


Fig. 1. The carboxy-terminal half of AhRR shows a repressor activity, which is sensitive to the HDAC inhibitor, TSA. (A) Schematic representation of luciferase reporter assay. (B) Transcriptional repression activity of AhRR. Hepa-1 cells were transiently transfected with the expression plasmids of GAL4DBD-AhRR and GAL-TK-Luc reporter gene containing three GAL binding sites. Cell extracts were prepared 48 h after transfection and used for luciferase assays. The fold repression is relative to the reporter gene alone. (C) Effects of TSA on AhRR-mediated transcriptional repression. The transfections were performed as described in (B), Cells were treated with increasing amounts (2, 4, and 8 ng/ml) of TSA 24 h after transfection and then, 12 h later, whole cell extracts were prepared and used for luciferase assays.

(478–555) did not significantly repress luciferase expression. These data localized a core region needed for transcriptional repression by AhRR to the sequence of amino acid 555–701.

To investigate how the repression activity of the AhRR fragment (342–701) functions in the TK promoter-driven reporter system, we used the HDAC inhibitor, TSA, which reversed the repression of reporter gene expression by the AhRR fragment (342–701) in a dose-dependent manner (Fig. 1C). These results suggest that the repression activity of the AhRR C-terminus is due to an HDAC activity. Since the C-terminal sequence of AhRR is well conserved among multiple mammalian species (Supplementary Fig. S1), we next searched for transcriptional corepressor, which interact with the AhRR C-terminus.

Isolation of ANKRA2 as a factor interacting with AhRR and interaction of AhRR with ANKRA2 and HDAC4 or HDAC5

To isolate a corepressor of AhRR, we performed a Cytotrap yeast two-hybrid screen with the C-terminal fragment of AhRR (342–701) used as bait (Fig. 2A) and isolated several clones including Dhx8, EB1, EB3, p21, Prostaglandin E receptor, EGF-containing fibrin-like extracellular matrix protein1, and ANKRA2. We chose ANKRA2 for further work in this paper, because ANKRA2 is reported to interact with HDAC4 and HDAC5 [18]. Recently, its mammalian paralogue, RFXANK has also been reported to interact with HDAC4 and HDAC5 [18] and to repress MHC class II promoter activation through association with HDAC4 and HDAC5 [19]. Taken together, these results suggest a potential role of ANKRA2 as mediator in transcriptional repression. ANKRA2 is a protein of 312 amino acids with consecutive 3 ankyrin repeats and the cDNA encoded a C-terminal fragment, amino acid 117–312 (Fig. 2A).

To address whether a physical interaction occurs between AhRR and ANKRA2, whole cell extracts from COS-7 cells cotransfected with expression plasmids for HA-tagged AhRR and FLAG-tagged ANKRA2 were used for *in vivo* coimmunoprecipitation studies. As expected, when the cell extracts were immunoprecipitated with an anti-FLAG antibody, AhRR was coimmunoprecipitated

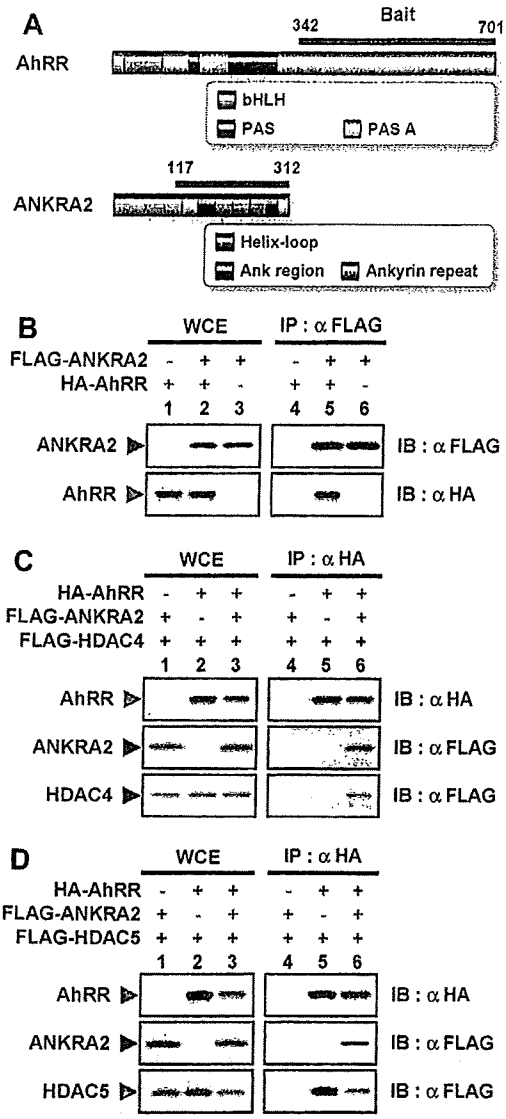


Fig. 2. Physical interaction of AhRR with ANKRA2 and HDAC4 or HDAC5. (A) Schematic diagrams of AhRR and ANKRA2. The carboxy-terminal half of AhRR was used as bait in a yeast two-hybrid screen. The AhRR bait interacted with a fragment (117–312 amino acid) of ANKRA2. ANKRA2 is a protein of 312 amino acids that contains an amino-terminal helix-loop-helix domain and four ankyrin repeats at the C terminus. (B) COS-7 cells were cotransfected with expression plasmids of HA-tagged AhRR and FLAG-tagged ANKRA2. Whole cell extracts were prepared 48 h after transfection and immunoprecipitated (IP) with anti-FLAG antibodies. Immunoprecipitates were analyzed by immunoblot with the indicated antibodies. Crude lysates were analyzed by immunoblot to control protein expressions (WCE). (C) COS-7 cells were cotransfected with expression plasmids of HA-tagged AhRR, FLAG-tagged ANKRA2 and either FLAG-tagged HDAC4 or HDAC5. Cell extracts were prepared 48 h after transfection and immunoprecipitated (IP) with anti-HA antibodies. Proteins were analyzed by immunoblot as described above.

with ANKRA2 (Fig. 2B, lane 5). We next asked if HDAC4 and HDAC5 also interact with AhRR. Whole cell extracts from COS-7 cells cotransfected with expression plasmids for HA-tagged AhRR and FLAG-tagged ANKRA2 together with either FLAG-tagged HDAC4 or FLAG-tagged HDAC5 were immunoprecipitated with an anti-HA antibody. HDAC4 was coimmunoprecipitated with AhRR only when ANKRA2 was cotransfected (Fig. 2C, lane 6). On the other hand, HDAC5 was also coimmunoprecipitated with AhRR even in the absence of ANKRA2, indicating that HDAC5 interacts with AhRR either directly or through ANKRA2 (Fig. 2D, lanes 5 and 6).

Depletion of ANKRA2 by siRNA attenuates transcriptional repression activity of AhRR

If ANKRA2 is a corepressor for AhRR, downregulation of the endogenous level of ANKRA2 by siRNA against ANKRA2 should reverse the transcriptional repression activity of AhRR. To confirm the requirement of ANKRA2 for the repression activity of AhRR, Hepa-1 cells were transiently transfected with expression plasmids for GAL-AhRR together with ANKRA2 siRNA or control siRNA. As shown in Fig. 3A, ANKRA2 siRNA transfection significantly reduced the expression of ANKRA2 mRNA. As expected, when the cells were treated with ANKRA2 siRNA, the repression activity of AhRR was significantly reversed as compared with that observed in cells treated with control siRNA (Fig. 3B).

To further investigate whether ANKRA2 is required for the endogenous AhRR/Arnt heterodimer to repress the XRE-driven transcription of endogenous target genes such as *CYP1A1*, we conducted RNA interference experiments in MEF cells. When MEF cells were transfected with ANKRA2 siRNA, endogenous ANKRA2 mRNA levels were significantly lowered both in the presence or absence of an AhR ligand, 3MC (Fig. 3C). Under normal conditions, CYP1A1 mRNA expression was barely detectable, but knockdown of ANKRA2 by ANKRA2 siRNA caused a small, but significant increase in basal CYP1A1 mRNA levels (Fig. 3D, lane 1). When treated with 3MC for 6 h, MEF cells inducibly expressed CYP1A1 mRNA to a similar level in the presence and absence of ANKRA2 siRNA (Fig. 3D, lane 2). These results suggest that in the silent state of MEF cells, the AhRR-ANKRA2 suppressor complex is involved in silencing the CYP1A1 basal expression, while under inducing conditions, ANKRA2 does not greatly affect the inducible expression of *CYP1A1* gene.

To confirm that the AhRR–ANKRA2 repressor complex silences the transcription of endogenous CYP1A1 in MEF cells, we used siRNA against AhRR. As previously reported in many other cell lines and tissues, AhRR mRNA and protein was constitutively expressed in MEF cells under normal conditions and was further enhanced in response to 3MC (Fig. 3E, lanes 1 and 2, and F). When MEF cells were transfected with AhRR siRNA, both mRNA and protein levels of AhRR were markedly downregulated (Fig. 3E, lanes 1 and 3, and F). In agreement with the result of the ANKRA2 siRNA experiment, treatment of AhRR siRNA increased CYP1A1 mRNA under normal

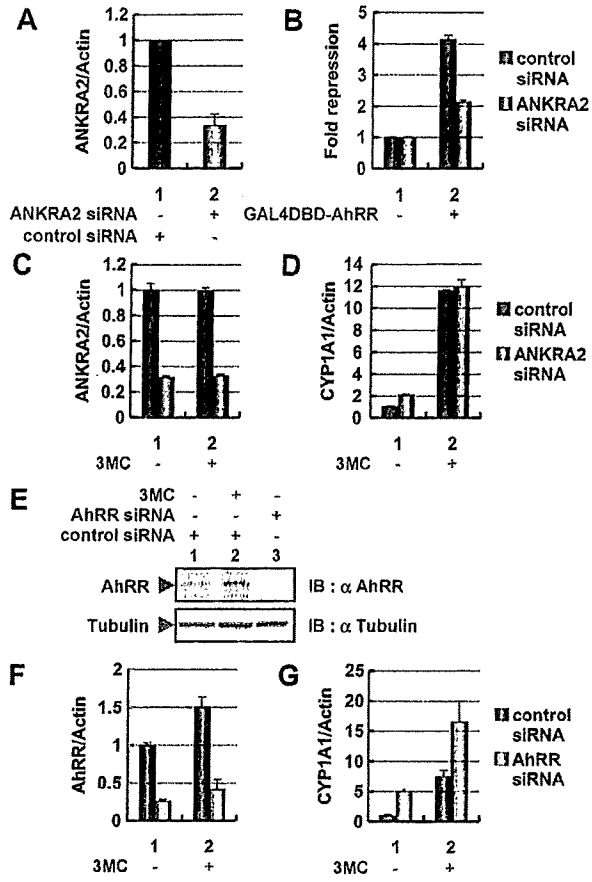


Fig. 3. Effects of ANKRA2 and AhRR siRNAs on the transcriptional repression activity of AhRR. (A) Hepa-1 cells were transiently transfected with ANKRA2 or control siRNA and 48 h after transfection, RNA were prepared from the transfected cells. ANKRA2 mRNA was quantified by RT-PCR method. (B) Hepa-1 cells were transiently transfected with GAL4DBD-AhRR and a reporter gene of GAL-TK-Luc, along with ANKRA2 siRNA or control siRNA. Cell extracts were prepared 48 h after transfection and used for luciferase assays. (C–G) MEF cells were transiently transfected with ANKRA2 siRNA (C and D) or AhRR siRNA (E–G) or control siRNA. After 48 h of transfection, cells were treated with 2 μM of 3MC or Me_2SO and then, 24 h later, cell extracts were prepared and indicated mRNA expression level was quantified using real time RT-PCR (C, D, E, and F) or immunoblot analysis (E).

conditions to even a higher extent than ANKRA2 siRNA treatment (Fig. 3G, lane 1). Interestingly, AhRR siRNA also further increased the induced expression of CYP1A1 mRNA in response to 3MC, as compared with the expression in cells treated with control siRNA, suggesting the existence of an ANKRA2-independent repression mechanism by AhRR (Fig. 3G, lane 2). As reported previously, AhRR may prevent AhR from forming a heterodimer with Arnt in a competitive manner, thereby blocking the binding of AhR to the XRE sequence in the absence of ANKRA2. Taken together, these results indicate that in a silent state of CYP1A1 expression under normal conditions, AhRR represses the expression of *CYP1A1* gene by binding the XRE sequence and recruiting ANKRA2, HDAC4 and/or HDAC5.

Discussion

We previously reported that AhRR repressed AhR transcription activity by competing with Arnt in AhR/Arnt heterodimerization, as well as by binding the XRE sequence in the promoter of CYP1A1 gene. Since Arnt has a weak transcription activity at its C-terminal end, it is possible that AhRR could have some inhibitory activity to compensate for Arnt transcription activity. In this report, we first demonstrated that the C-terminus (555–701 a.a.) of AhRR shows a transcription inhibitory activity which was sensitive to the HDAC inhibitor, TSA, suggesting that AhRR which binds the XRE sequence recruits a HDAC protein. To isolate factors that interact with the C-terminal sequence of AhRR, we utilized the Cytotrap yeast two-hybrid screening method using the C-terminal sequence of AhRR as bait, resulting in isolation of a fragment (113–312 a.a.) of ANKRA2 consisting of 312 amino acids and containing three ankyrin (ANK) repeats. The isolated ANKRA2 fragment was shown to physically interact with AhRR (Fig. 2B). ANK repeats are one of the most common protein sequence motifs mediating protein–protein interactions, but they have not been clarified to bind any specific amino acid sequence or structure. Rather, they are thought to bind a variety of proteins through adaptive alterations in their binding surface features and in the domain size of the ANK repeat by sequence duplication or deletion [20]. Recent studies have demonstrated that ANKRA2 also interacts with megalin [21] and the α-subunit of rat large-conductance Ca^{2+}-activated K^+ channel (rSlo) [22]. According to Rader et al. [21], the C-terminus (177–312 a.a.) of ANKRA2 interacts with a proline-rich motif (PXXPXXP) within the 19 amino acid sequence of the magalin tail, and Lim and Park [22] have shown that 52–150 a.a. of ANKRA2 interacts with the C-terminal end (1119–1210 a.a.) of rSlo Channel. Since there is no apparent sequence similarity in the ANKRA2-interacting domains of megalin, rSlo and AhRR, the precise molecular mechanisms how ANKRA2 interacts with these proteins remain to be investigated.

Our coimmunoprecpitation experiments have revealed that HDAC4 interacts with AhRR in an ANKRA2-dependent manner. HDAC5 may interact with AhRR either directly or via ANKRA2, when the previous report is taken into account [18]. It remains to be studied how HDAC4 and HDAC5 are recruited on the surface of the AhRR and ANKA2 complex, reciprocally or simultaneously. ANKRA2 is also abundantly and ubiquitously expressed in various tissues of mice [21] and cultured cells such as Hepa-1, HeLa and MEF cells used in this study (data not shown). For functional analysis of ANKRA2 and AhRR, we used siRNA to knock down gene expres-

sion. In Hepa-1 cells, repression of TK promoter-driven luciferase activity by GAL4DBD-AhRR was reversed by the addition of ANKRA2 siRNA, indicating that the repression activity of AhRR required ANKRA2. Treatment of MEF cells with ANKRA2 siRNA significantly activated the expression of *CYP1A1* under normal conditions. In contrast, the siRNA treatment had apparently no effect on the enhanced CYP1A1 expression in response to the inducer. These results suggest that under normal conditions, a silent state of *CYP1A1* gene expression is not merely due to the lack of a transcription activator, but resulted from negative regulation by a heterodimer of AhRR and Arnt, which recruits ANKRA2. This observation was substantiated by the experiments using AhRR siRNA, which significantly enhanced the expression of *CYP1A1* gene under normal conditions to even a higher level than did ANKRA2 siRNA under the non-inducing conditions. The greater effect of AhRR siRNA on CYP1A1 expression becomes more pronounced under inducing conditions. These observations could be explained by a two step inhibitory mechanism. First, AhRR inhibits the transcription activity of AhR in an ANKRA2-independent manner, by competing with AhR for forming a heterodimer with Arnt and binding the XRE sequence, as reported previously. Next, AhRR bound to the XRE sequence recruits ANKRA2 and HDAC4 and/or HDAC5 for more efficient repression. In the presence of the inducer 3MC, AhRR synthesis is accelerated so that the AhRR siRNA treatment displays a greater effect on the inducible expression of CYP1A1 than ANKRA2 siRNA. Recently, we have found that the silent state of CYP1A1 is actually negatively regulated by the AhRR system in macrophages (unpublished data). We will be investigating how this silencing mechanism involving AhRR and ANKRA2 functions in different cell types and how it affects target genes other than *CYP1A1* under normal conditions.

Acknowledgments

We thank Mrs. Y. Nemoto for clerical work. This work was funded in part by Solution Oriented Research for Science and Technology, Japan Science and Technology Agency and by a Grant for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.09.131.

References

[1] J. Mimura, Y. Fujii-Kuriyama, Functional role of AhR in the expression of toxic effects by TCDD, Biochim. Biophys. Acta 1619 (2003) 263–268.

[2] A. Fujisawa-Sehara, K. Sogawa, M. Yamane, Y. Fujii-Kuriyama, Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P-450c gene: A similarity to glucocorticoid regulatory elements, Nucleic Acids Res. 15 (1987) 4179–4191.

[3] N.M. Brown, P.A. Manzolillo, J.X. Zhang, J. Wang, C.A. Lamartiniere, Prenatal TCDD and predisposition to mammary cancer in the rat, Carcinogenesis 19 (1998) 1623–1629.

[4] A. Gibbons, Dioxin tied to endometriosis, Science 262 (1993) 1373.

[5] J. Mimura, K. Yamashita, K. Nakamura, M. Morita, T.N. Takagi, K. Nakao, M. Ema, K. Sogawa, M. Yasuda, M. Katsuki, Y. Fujii-Kuriyama, Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the ah (dioxin) receptor, Genes Cells 2 (1997) 645–654.

[6] Y. Shimizu, Y. Nakatsuru, M. Ichinose, Y. Takahashi, H. Kume, J. Mimura, Y. Fujii-Kuriyama, T. Ishikawa, Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor, Proc. Natl. Acad. Sci. USA 97 (2000) 779–782.

[7] F. Ohtake, K. Takeyama, T. Matsumoto, H. Kitagawa, Y. Yamamoto, K. Nohara, C. Tohyama, A. Krust, J. Mimura, P. Chambon, J. Yanagisawa, Y. Fujii-Kuriyama, S. Kato, Modulation of oestrogen receptor signalling by association with the activated dioxin receptor, Nature 423 (2003) 545–550.

[8] T. Baba, J. Mimura, N. Nakamura, N. Harada, M. Yamamoto, K. Morohashi, Y. Fujii-Kuriyama, Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction, Mol. Cell. Biol. 25 (2005) 10040–10051.

[9] B.D. Abbott, J.E. Schmid, J.A. Pitt, A.R. Buckalew, C.R. Wood, G.A. Held, J.J. Diliberto, Adverse reproductive outcomes in the transgenic ah receptor-deficient mouse, Toxicol. Appl. Pharmacol. 155 (1999) 62–70.

[10] J. Mimura, M. Ema, K. Sogawa, Y. Fujii-Kuriyama, Identification of a novel mechanism of regulation of ah (dioxin) receptor function, Genes Dev. 13 (1999) 20–25.

[11] T. Baba, J. Mimura, K. Gradin, A. Kuroiwa, T. Watanabe, Y. Matsuda, J. Inazawa, K. Sogawa, Y. Fujii-Kuriyama, Structure and expression of the ah receptor repressor gene, J. Biol. Chem. 276 (2001) 33101–33110.

[12] Y. Tsuchiya, M. Nakajima, S. Itoh, M. Iwanari, T. Yokoi, Expression of aryl hydrocarbon receptor repressor in normal human tissues and inducibility by polycyclic aromatic hydrocarbons in human tumor-derived cell lines, Toxicol. Sci. 72 (2003) 253–259.

[13] M. Korkalainen, J. Tuomisto, R. Pohjanvirta, Primary structure and inducibility by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) of aryl hydrocarbon receptor repressor in a TCDD-sensitive and a TCDD-resistant rat strain, Biochem. Biophys. Res. Commun. 315 (2004) 123–131.

[14] S.I. Karchner, D.G. Franks, W.H. Powell, M.E. Hahn, Regulatory interactions among three members of the vertebrate aryl hydrocarbon receptor family: AHR repressor, AHR1, and AHR2, J. Biol. Chem. 277 (2002) 6949–6959.

[15] B.R. Evans, S.I. Karchner, D.G. Franks, M.E. Hahn, Duplicate aryl hydrocarbon receptor repressor genes (ahrr1 and ahrr2) in the zebrafish danio rerio: Structure, function, evolution, and AHR-dependent regulation in vivo, Arch. Biochem. Biophys. 441 (2005) 151–167.

[16] M. Ema, M. Suzuki, M. Morita, K. Hirose, K. Sogawa, Y. Matsuda, O. Gotoh, Y. Saijoh, H. Fujii, H. Hamada, Y. Fujii-Kuriyama, cDNA cloning of a murine homologue of drosophila single-minded, its mRNA expression in mouse development, and chromosome localization, Biochem. Biophys. Res. Commun. 218 (1996) 588–594.

[17] S. Mizushima, S. Nagata, pEF-BOS, a powerful mammalian expression vector, Nucleic Acids Res. 18 (1990) 5322.

[18] A.H. Wang, S. Gregoire, E. Zika, L. Xiao, C.S. Li, H. Li, K.L. Wright, J.P. Ting, X.J. Yang, Identification of the ankyrin repeat proteins ANKRA and RFXANK as novel partners of class IIa histone deacetylases, J. Biol. Chem. 280 (2005) 29117–29127.

[19] T.A. McKinsey, K. Kuwahara, S. Bezprozvannaya, E.N. Olson, Class II histone deacetylases confer signal responsiveness to the ankyrin-repeat proteins ANKRA2 and RFXANK, Mol. Biol. Cell 17 (2006) 438–447.
[20] S.G. Sedgwick, S.J. Smerdon, The ankyrin repeat: A diversity of interactions on a common structural framework, Trends Biochem. Sci. 24 (1999) 311–316.
[21] K. Rader, R.A. Orlando, X. Lou, M.G. Farquhar, Characterization of ANKRA, a novel ankyrin repeat protein that interacts with the cytoplasmic domain of megalin, J. Am. Soc. Nephrol. 11 (2000) 2167–2178.
[22] H.H. Lim, C.S. Park, Identification and functional characterization of ankyrin-repeat family protein ANKRA as a protein interacting with BKCa channel, Mol. Biol. Cell 16 (2005) 1013–1025.

水環境学会誌 Journal of Japan Society on Water Environment Vol.30, No.2, pp.89-94 (2007)
〈論 文—Original Paper〉

PRTR化学物質の各種核内受容体に対する結合性

井上大介*　松井久恵*　清和成*
楊敏**　胡建英***　荒金淳†
廣辻淳二†　西川淳一††　池道彦*

Binding Affinity of PRTR Chemicals to Various Human Nuclear Receptors

**Daisuke INOUE*, Hisae MATSUI*, Kazunari SEI*,
Min YANG**, Jianying HU***, Jun ARAGANE†,
Junji HIROTSUJI†, Jun-ichi NISHIKAWA†† and Michihiko IKE***

* Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

** State Key Lab of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

*** College of Environmental Science, Peking University, Beijing 100871, China

† Advanced Technology R&D Center, Mitsubishi Electric Co., 8-1-1 Tsukaguchi-Honmachi, Amagasaki, Hyogo 661-8661, Japan

†† Department of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

Abstract

Since the 1990s, population decreases, reproductive anomalies and malformations of highly aquatic animals have been increasingly reported. One possible cause is considered to be endocrine disruptive effects induced by environmental contaminants through a direct interaction with nuclear receptors, not only with steroid hormone receptors but also with other ones. In this study, we examined the binding affinities of 20 chemicals, which are registered in the Japanese Pollutant Release and Transfer Register (PRTR) and have been abundantly discharged into aquatic environments to eight human nuclear receptors and assessed their potential endocrine disruptive effects. Of the 20 PRTR chemicals tested, nonylphenol diethoxylate, telephthalic acid (TPA), and linear dodecyl-benzensulfonate (DBS) bound to at least two receptors at high concentrations. TPA and DBS enhanced the activities of both retinoic acid receptor (RAR) γ and vitamin D receptor (VDR) in a dose-dependent manner. This suggests that TPA and DBS may disturb the vitamin D endocrine functions mediated by a VDR-VDR homodimer or a VDR-RAR heterodimer. Also, our results indicate that endocrine disruptors unsuspected under the current assessment criteria could potentially bind to various nuclear receptors and disrupt endocrine systems mediated by such receptors.

Key words: aquatic environment, endocrine disruptive effect, nuclear receptor, PRTR chemical

1．はじめに

1990年代から，魚類や両生類など，水への依存度の高い野生生物種において，個体数の減少，生殖異常，形態異常の発生が数多く報告されている[1-6]。このような危機的状況をもたらした原因の一つは，環境中に放出された人工化学物質のホルモン様作用に起因する正常な内分泌バランスの攪乱にあるとされている[7]。内分泌攪乱化学物質（endocrine disruptors；EDs）の内分泌機能への影響発現には，核内受容体（nuclear receptor；NR）を介するメカニズムと介さないメカニズムが存在するが，その大部分はNRへの直接作用によると考えられている[8]。NRの中には，ヒトを含め，異なる生物種間で高い保存性を示すものもあることから[9]，EDsによる内分泌機能攪乱に伴う悪影響

＊　大阪大学大学院工学研究科環境・エネルギー工学専攻　〒565-0871　大阪府吹田市山田丘2-1
＊＊　中国科学院生態環境研究中心　Beijing 100085, China
＊＊＊　北京大学環境学院　Beijing 100871, China
†　三菱電機㈱先端技術総合研究所　〒661-8661　兵庫県尼崎市塚口本町8-1-1
††　大阪大学大学院薬学研究科　〒565-0871　大阪府吹田市山田丘1-6

がヒトにも生じる可能性があるものと推測される。

従来，EDsに関する研究は，主にエストロゲン受容体（estrogen receptor；ER）などの性ホルモン受容体や甲状腺ホルモン受容体（thyroid hormone receptor；TR）を中心に進められてきた。しかし近年，ヒトのNRファミリーに48種類の受容体が存在することが断定され[10]，EDsの作用点が性ホルモン受容体やTR以外のNRにもある可能性が議論されるようになった[11,12]。例えば，プラスチックの可塑剤等に使用されるフタル酸ジエステルの生体内代謝物であるフタル酸モノエステルによる雌生殖毒性等の生態毒性にはペルオキソーム増殖剤活性化受容体（peroxisome proliferator-activated receptor；PPAR）[13,14]が，また有機スズ化合物によるイボニシ貝のインポセックス発達の促進にはレチノイドX受容体（retinoid X receptor；RXR）[15]が関与していることが示唆されている。また，地下水汚染物質の一つ，トリクロロエチレンの生体内代謝物であるトリクロロ酢酸とジクロロ酢酸による肝臓癌とPPARαの関連性も指摘されている[16,17]。これらの新たな科学的事実から，化学物質の内分泌攪乱活性を評価するためには，様々なNRに対する作用を網羅的に検討することが重要であるといえる。すなわち，これまでに性ホルモン受容体やTRについて内分泌攪乱作用がないと判定された化学物質を含め，多くの人工化学物質のNRに対する作用を検討していくことが必要である。本研究では，水環境中に大量に放出されている化学物質のヒトNRに対する結合性を調査し，潜在的な内分泌攪乱作用の可能性を探った。被検物質には，環境汚染物質排出移動登録（pollutant release and transfer register；PRTR）制度の第1種指定化学物質に含まれ，公共用水域への年排出量が15tを上回る有機化学物質群の中から20種類を選定した。被検物質のNRに対する結合性の評価は，ヒトNRのうちERα，TRα，ビタミンA受容体（retinoic acid receptor；RAR）γ，RXRα，ビタミンD受容体（vitamin D receptor；VDR），PPARα/γ/δを対象として，*in vitro*でNRとコアクチベーターの相互作用を検出できるハイスループットスクリーニング法であるCoA-BAP（coactivator-bacterial alkaline phosphatase）法[18]を用いて実施した。

2．方　　法

2.1 化学物質

標準リガンドとして，ERαには17β-エストラジオール（E2），TRαには3,3',5-トリイオド-L-チロニン（T3），RARγには*all-trans*-レチノイン酸（retinoic acid；RA），RXRαには9-*cis* RA，VDRには1α,25-ジヒドロキシビタミンD3（1,25(OH)$_2$D3），PPARαにはGW7647，PPARγにはRosiglitazone，PPARδにはGW501516を用いた。

PRTR制度で第1種指定化学物質に指定され，公共用水域への年間排出量15t以上で，水より蒸気圧が低い有機化学物質群の中から20種類の化学物質（**Table 1**）を被検物質として選定した。直鎖ドデシルベンゼンスルホン酸ナトリウム（linear dodecyl-benzenesulfonate；DBS）は，直鎖アルキルベンゼンスルホン酸塩（linear-alkylbenzenesulfonate；LAS）の代表として選出した。また，ノニルフェノールジエトキシレート（nonylphenol diethoxylate；NP2EO）は，ノニルフェノールポリエトキシレート（nonylphenol polyethoxylate；

Table 1 Binding affinity of 20 PRTR chemicals for various nuclear receptors[a]

No.	Compound	ERα	TRα	RARγ	RXRα	VDR	PPARα	PPARγ	PPARδ
1	Ethylene glycol	–	–	–	–	–	–	–	–
2	N,N-Dimethylformamide	–	–	–	–	–	–	–	–
3	Nonylphenol diethoxylate (NP2EO)	++	–	–	–	–	–	+	–
4	Thiourea	–	–	–	–	–	–	–	–
5	ε-Caprolactam	–	–	–	–	–	–	–	–
6	Ethylenediaminetetraacetic acid	–	–	–	–	–	–	–	–
7	Terephthalic acid (TPA)	–	–	++	–	++	–	+	–
8	Diethylenetriamine	–	–	–	–	–	–	–	–
9	Toluene	–	–	–	–	–	–	–	–
10	1,4-Dioxane	–	–	–	–	–	–	–	–
11	Methacrylic acid	–	–	–	–	–	–	–	–
12	2-Aminoethanol	–	–	–	–	–	–	–	–
13	Acrylic acid	–	–	–	–	–	–	–	–
14	Linear dodecyl-benzenesulfonate (DBS)	–	+	++	–	++	–	+	–
15	1,3-Dichloro-2-propanol	–	–	–	–	–	–	–	–
16	Hexamethylenediamine	–	–	–	–	–	–	–	–
17	*p*-Xylene	–	–	–	–	–	–	–	–
18	Aniline	–	–	–	–	–	–	–	–
19	Pyridine	–	–	–	–	–	–	–	–
20	Phenol	–	–	–	–	–	–	–	–

[a] ++, the lowest detectable effective concentrations of tested chemicals were 10^3 to 10^5 times as much as that of the cognate ligand; +, 10^6 to 10^8 times; –, not detected.