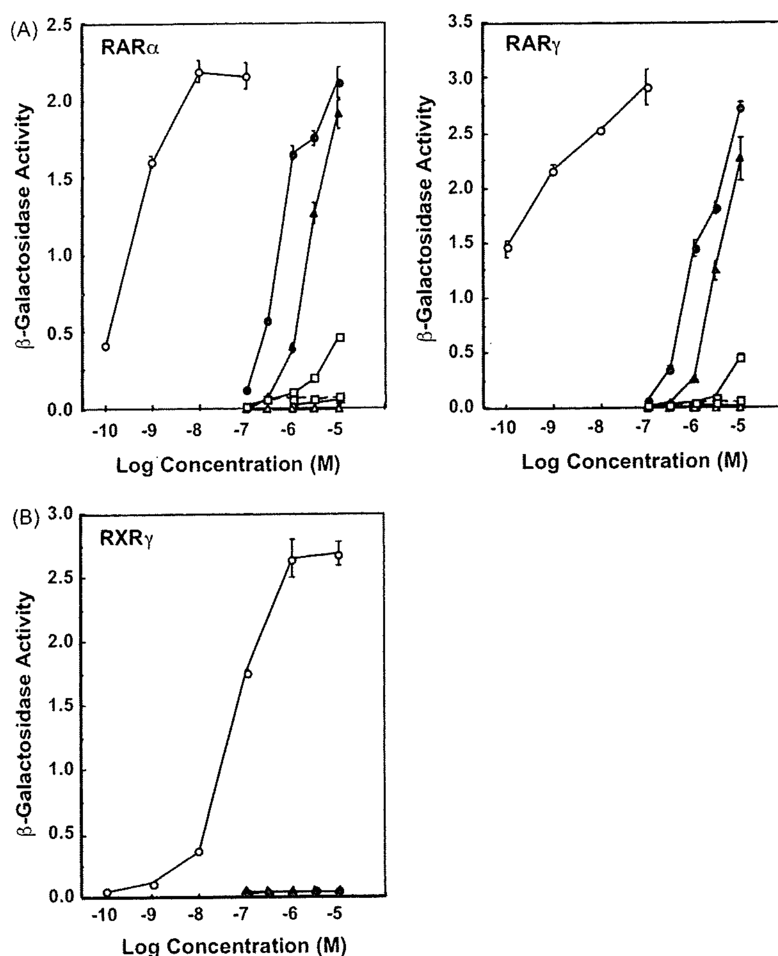

Fig. 2 – Yeast two-hybrid assay for RARα or RARγ activity. Yeast strain Y190 was transformed with GAL4 activation domain fused to TIF2 and GAL4 DNA binding domain fused to agonist-binding domain of RARα or RARγ. Chemicals were added to yeast cultures in doses ranging from 10^{-10} to 10^{-5} M. Following 4 h incubation the cultures were then assayed for β-galactosidase activity. Open circle in (A) and (B): atRA and 9-cis RA, respectively, closed circle: β-cryptoxanthin, open triangle: astaxanthin, closed triangle: lultein, open square: β-carotene, closed square: zeaxanthin, open square with broken line: lycopene. The values are represented as means ± S.E. of three determinants from a representative of three independent experiments, which showed similar results.

cryptoxanthin is a more potent ligand than lutein. Simultaneously, it has been shown that β-carotene, zeaxanthin, astaxanthin, and lycopene failed to show similar activity. An RAR pan-antagonist, LE540, completely abolished the β-crypotoxanthin- or lutein-dependent interaction between GAL4-RAR LBD and GAL4-TIF2 in the yeast two-hybrid assay, indicating that β-cryptoxanthin and lutein serve as an RAR agonist. The agonist activity was also supported by the finding that β-cryptoxanthin induces ABCA1 and ABCG1 mRNAs and ABCA1 protein in macrophages. To eliminate the possibility that metabolites or degradation products of β-cryptoxanthin act as RAR agonist, the binding assay was performed in cell-free CoA-BAP system. β-Cryptoxanthin exhibited binding activity against RAR LBD in a dose-dependent manner similar with the finding in the yeast two-hybrid assay. These data led to the conclusion that β-cryptoxanthin and lutein act as natural RAR agonists and may have preventive effects on atherosclerosis and restenosis.

β-Carotene is known to be cleaved in the intestinal mucosa at higher efficiency than any other organs. A responsible enzyme for cleaving β-carotene is β-carotene 15,15′-dioxygenase [36], which has a low level of activity toward provitamin A sources aside from β-carotene. The resulting all-*trans*-retinol (vitamin A) is first esterified to give an all-*trans*-retinyl ester, which is stored in the liver. In plasma, the vitamin A concentration is usually maintained at about 1 μM and the biologically active retinoid, atRA, is produced as the need arises in cells where the metabolic enzymes, aldehyde dehydrogenase and retinal dehydrogenase exist. The plasma concentration of atRA is therefore maintained at around a 12 nM level [37] and about 15% of this form is converted to 9-cis retinoic acid [38]. When considering the individual carotinoid and xanthopyll concentrations in plasma, β-cryptoxanthin has been shown to reach a concentration than other major carotenoids and xanthophylls, including β-carotene, α-carotene, lycopene, lutein, and zeaxanthin, assuming that the

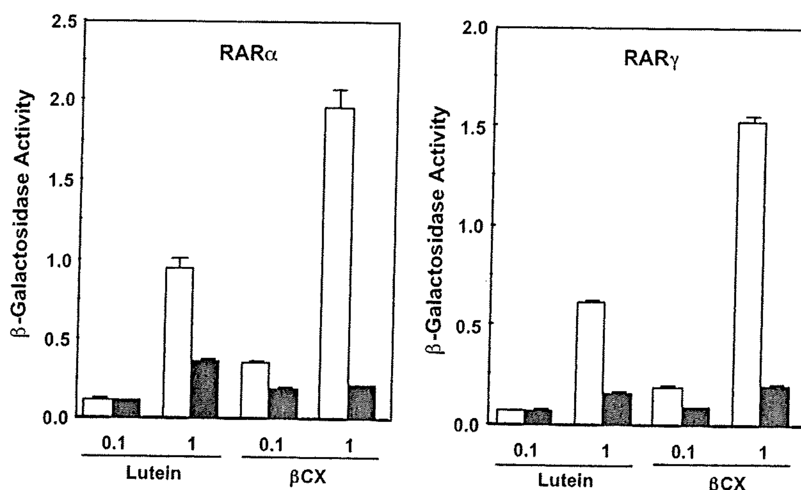

Fig. 3 – Effect of RAR pan-antagonist, LE540, on the transcriptional activity of β-cryptoxanthin or lutein against RARα or RARγ. Following 30 min incubation of 10 μM LE540 with yeast cultures, β-cryptoxanthin or lutein was added to yeast cultures at the indicated concentrations (μM) and incubated for 4 h. The cultures were then assayed for β-galactosidase activity. Open column: without LE540, closed column: with LE540. The values are represented as means ± S.E. of three determinants from a representative of three independent experiments, which showed similar results.

equal amounts of each of these carotenoid is present in the diet [39]. In actuality, β-cryptoxanthin reaches to 0.4–1.1 μM concentrations in the plasma after the intake of orange juice rich in cryptoxanthin [40,41]. In addition, the β-cryptoxanthin concentration in adipose tissue is also higher than that of other carotenoids and that the tissue distribution of β-cryptoxanthin is definitely different from that of atRA. The results of this study have shown that a 5×10^{-7} M of β-cryptoxanthin is sufficient to transactivate RAR and act as an RAR agonist, although the RAR-binding affinity of β-cryptoxanthin is three orders of magnitude lower than that of atRA. These results led to the hypothesis that the difference in the pharmacokinetics between β-cryptoxanthin and atRA makes β-cryptoxanthin an RAR agonist with physiological effects distinct from atRA. Although the preventive effects of vegetable and fruit intake against cardiovascular diseases have been considered based the known anti-oxidative effects of carotenoids found in vegetables and fruits, the result presented here strongly suggest that a β-cryptoxanthin signaling mechanism via RAR/RXR heterodimer contributes to the preventive effects by enhancing the expression of anti-atherogenic molecules such as ABCA1 and ABCG1.

This study did not reveal a structure–activity relationship that can explain why β-cryptoxanthin exhibited better binding activity to RAR in comparison to the other carotenoids and xanthopylls tested in this study. However, this difference may potentially be explained based upon the polarity and hydrophobicity properties of these compounds. It is reported that

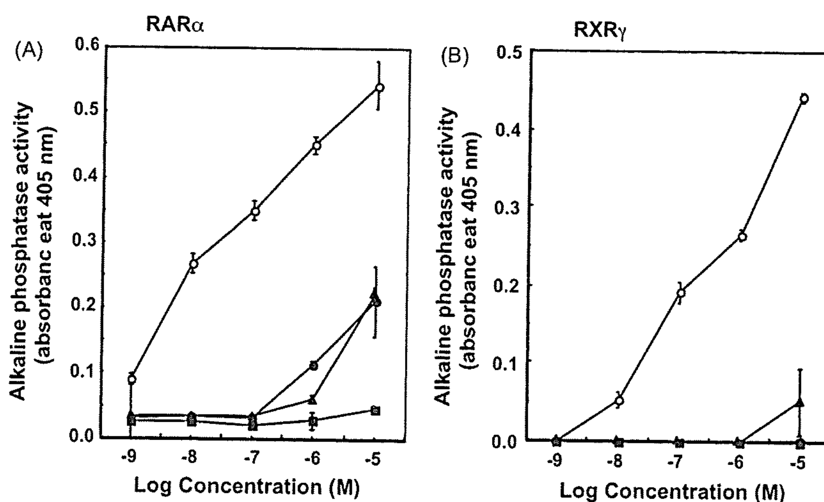

Fig. 4 – Binding activity of β-cryptoxanthin and lutein against RARγ in *in vitro* CoA-BAP system. Agonist-dependent interactions between GST-RARγ and TIF2-BAP were determined as alkaline phosphatase activity according to the method described in Section 2. Open circle in (A) and (B): atRA and 9-cis RA, respectively, closed circle: β-cryptoxanthin, closed triangle; lutein, closed square: zeaxanthin. The values are represented as means ± S.E. of three determinants.

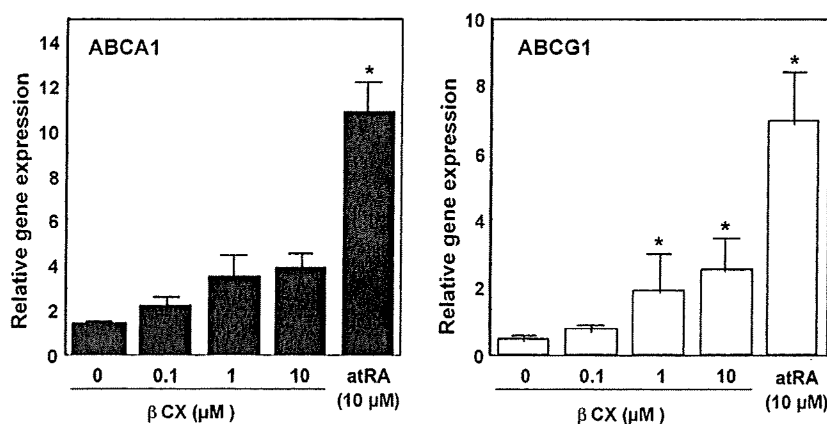

Fig. 5 – Effect of β-cryotoxanthin on induction of ABCA1 or ABCG1 mRNA in mouse peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with β-cryptoxanthin or atRA at the indicated concentrations for 8 h. mRNA levels of ABCA1 and ABCG1 were determined by quantitative RT-PCR as described in Section 2. Data were normalized using the β-actin levels and are expressed relative to the cells treated with vehicle (DMSO). The values are represented as means ± S.D. of 3–4 culture dishes. Statistically significant differences from the cells treated with vehicle are indicated followed by Dunnett's test ($^*p < 0.05$).

lutein and zeaxanthin, which are dipolar xanthophylls, appear to adopt an orientation mainly perpendicular to membrane surfaces because the hydrophilic groups at the opposite ends of the molecule are attracted to the polar zones of the membrane at the membrane surfaces [42]. For carotenes lacking such polar groups, such as β-carotene and lycopene, orientation in the lipid membrane environment seems to be exclusively governed by van der Waals interactions with the hydrocarbon acyl chains of lipid molecules thereby resulting

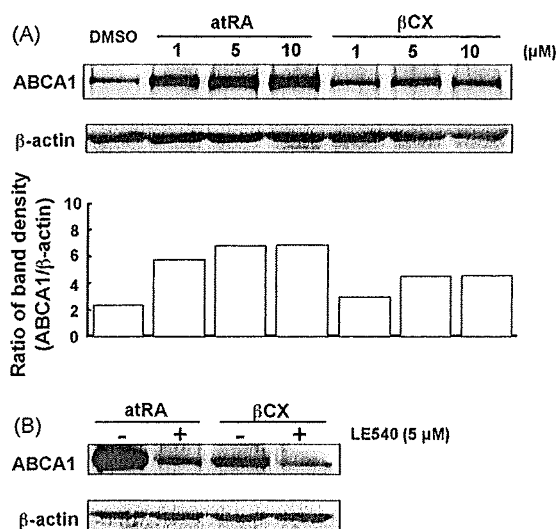

Fig. 6 – Induction of ABCA1 protein by β-cryptoxanthin. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with increasing concentrations of β-cryptoxanthin or atRA for 20 h in the absence (A) or presence (B) of an RAR pan-antagonist LE540 (5 μM). ABCA1 protein was determined by Western blot analysis as described in Section 2. The figure is a representative of three independent experiments, which show similar results.

in random orientations of these carotenoid in membranes. In the case of β-cryptoxanthin, a monopolar xanthophyll, the hydrophilic portion seems to be attracted to the membrane surface with hydrophobic end floating in the core of the membrane [43]. In fact, although RAR does not exist in membrane, such difference in molecular polarity may influence the interaction between ligands and RAR in RAR ligand-binding pocket. Recently, a zeaxanthin-binding protein was isolated and identified as a Pi isoform of glutathione S-transferase [44]. Thus, there is a possibility that a β-cryptoxanthin-specific protein exists and transactivates RAR in a ligand-dependent manner. Of particular interest from the present work is that β-carotene failed to show any ligand activity at physiological or pharmacological concentrations. In addition, it has been reported that the carboxyl group of retinoid cannot be replaced by typical bioisosteric functional groups, such as sulfonamide and tetrazole [45]. This suggests that β-cryptoxanthin may possess a structural uniqueness as a RAR ligand.

To date, there are only a limited numbers of reports concerning the biological activities of β-cryptoxanthin. Among them, β-cryptoxanthin has been found to possess a unique anabolic effect on bone calcification [46], stimulation of bone formation, and inhibition of bone resorption [47]. The authors of those resports denied the possibility that β-cryptoxanthin shows such effects via RAR activation, although the evidence is not completely clear. However, Lian et al. reported that β-cryptoxanthin suppresses the growth of human bronchial cells by upregulating RARβ expression [48]. They also indicated that β-cryptoxanthin can transactivate RAR-mediated transcription activity of the retinoic acid response element. However, they concluded that the metabolites of β-cryptoxanthin might induce RARβ expression based upon the need for a higher effective dose of β-cryptoxanthin than atRA for the induction of RARβ expression or transactivation of RARs. In contrast, the use of the CoA-BAP system employed in the present study to assay the ligand

activity against RARα reduces the potential that metabolites or degradation products of β-cryptoxanthin are responsible for such activity. Furthermore, in yeast two-hybrid assay, β-cryptoxanthin induced β-galactosidase via RARs at the concentration of 5×10^{-7} M. Furthermore, the RAR pan-antagonist, LE540, effectively inhibited β-galactosidase induction by β-cryptoxanthin. Taken together, these results indicate that β-cryptoxanthin itself, not its metabolites, acts as an RAR ligand and stimulates RAR-mediated transcription activity at physiological concentrations.

Considering that the prevalence of acute and chronic side effects has limited wide applicability of vitamin A and its retinoic acid derivatives as therapeutic drugs of choice, novel natural ligands against RAR should pave the way for not only understanding the mechanism underlying the pleiotropic effects of RA, but also developing the therapeutic agents with fewer side effects for atherosclerosis, cancer and other related conditions. In this respect, β-cryptoxanthin appears to be a molecule that warrants further investigation.

Recent epidemiological studies reveal that a moderate increase in β-cryptoxanthin intake is associated with a reduced risk of developing rheumatoid arthritis and lung cancer [49,50]. The results reported here led to the hypothesis that β-cryptoxanthin is capable of stimulating differentiation of lung cancer cells and modulating immune response through Th2 cells via RAR. Epidemiologic studies indicate that appropriate vegetable and fruit intake may be helpful in preventing cancer and cardiovascular diseases. Given the fact that vegetables and fruits contain β-cryptoxanthin, this molecule may indeed be the source of that anti-atherogenic and anti-tumor activity by acting through an RAR signaling mechanism.

In conclusion, the present study has shown that β-cryptoxanthin, a xanthophyll, exhibits agonist activity against RAR as well as exhibiting anti-atherogenic effect on macrophages by inducing ABCA1 and G1 expressions. Since natural RAR ligands other than retinol metabolites have not previously been found, β-cryptoxanthin, a provitamin A carotenoid that exhibits RAR agonist activity and acts as an RAR agonist which has hydrophobicity and exhibits a metabolic fate different from atRA, should be potential candidate for preventive or therapeutic agents against cardiovascular diseases.

Acknowledgments

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Changes in central dopaminergic systems and morphine reward by prenatal and neonatal exposure to bisphenol-A in mice: evidence for the importance of exposure period

Minoru Narita, Kazuya Miyagawa, Keisuke Mizuo, Takuya Yoshida & Tsutomu Suzuki

Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, Japan

ABSTRACT

Bisphenol-A has been extensively evaluated for toxicity in a variety of tests as the most common environmental endocrine disruptors. In a previous study, we reported that exposure to bisphenol-A affects the development of the central dopaminergic system in the mouse limbic area. The present study was undertaken to investigate the relationship between the developmental toxicity of bisphenol-A and its exposure period. The exposure to bisphenol-A during either organogenesis or lactation, but not implantation and parturition, significantly enhanced the morphine-induced hyperlocomotion and rewarding effects. Furthermore, exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These results indicate that both organogenesis and lactation are more sensitive to the bisphenol-A-induced developmental neuronal toxicology than any other periods. In conclusion, the present data suggest that the organogenesis and lactation are the most important period to cause the alternation of dopaminergic system by bisphenol-A exposure in the mouse.

Keywords Bisphenol-A, endocrine disruptor, exposure periods, hyperlocomotion, morphine, rewarding effect.

Correspondence to: Minoru Narita, Tsutomu Suzuki, Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2-4-41 Ebara, Shinagawaku, Tokyo 142-8501, Japan. E-mail: narita@hoshi.ac.jp; suzuki@hoshi.ac.jp

INTRODUCTION

Many aquatic ecosystems might be polluted with bisphenol-A, a possible endocrine disruptor that is widely used as a monomer for the production of plastics, resins and coatings (Staples *et al.* 1998). The global mass production is 1.7 million tons in the world and 0.35 million tons in Japan (reported by the BPA Safety Committee of Japanese Manufactures 2002) and the chemical is widely used all over the world. Bisphenol-A is a global environmental contaminant, used in polycarbonate plastics, and is reported to display weak estrogenic activity both *in vitro* and *in vivo* (Krishnan *et al.* 1993; Ashby & Tinwell 1998; Gould *et al.* 1998; Kuiper *et al.* 1998). The bioactivity of bisphenol-A is 1000–15 000 times lower than 17β-estradiol.

It has been reported that administration of bisphenol-A to pregnant mice on gestation days at a dose that is within the range typical of the environmental exposure of humans produces significant changes in the postnatal growth rate and brings on early puberty in these mice (Howdeshell *et al.* 1999). Bisphenol-A also inhibits the differentiation of oligodendrocyte precursor cells induced by exposure to thyroid hormone (Seiwa *et al.* 2004). These results support the idea that bisphenol-A may cause toxicity in the developmental process.

Recently, we have demonstrated that prenatal and neonatal exposure to bisphenol-A markedly enhances the rewarding effects induced by drugs of abuse, such as methamphetamine (Suzuki *et al.* 2003) and morphine (Mizuo *et al.* 2004a). In addition, prenatal and neonatal exposure to bisphenol-A enhances central dopamine D_1 receptor function (Suzuki *et al.* 2003) and attenuates dopamine D_3 receptor function (Mizuo *et al.* 2004b) in mice. These findings indicate that exposure to bisphenol-A may cause alterations in dopaminergic neurotransmission in the central nervous system resulting in the enhancement of drug reward. The aim of the present

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study was to further investigate the relationship between the effects of bisphenol-A on dopamine-related behaviors and the bisphenol-A exposure period, and to determine the most sensitive period in prenatal and neonatal exposure to bisphenol-A in mice.

MATERIALS AND METHODS

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering.

Animals

All experiments were performed using male ddY mice that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd, Osaka, Japan). Adult female mice were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 2 mg of bisphenol-A/g of food during the period of implantation [I-2; embryonic day (ED) 0–7], organogenesis (O-2; ED 7–14), parturition (P-2; ED 14–20) and lactation (L-2; postnatal day 0–20). Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, animals did not show weight loss or disrupted maternal behaviors. In addition, the pups did not show weight loss or decrease of birth rate. All experiments used mice aged 7–9 weeks.

Place conditioning

Place conditioning was conducted as previously described (Suzuki *et al.* 1991; Narita, Funada & Suzuki 2001). The apparatus was a shuttle box (15 × 30 × 15 cm: width × length × height), which was made of an acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is black with a smooth floor to create equally preferred compartments. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injection. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. Conditioning sessions (3 days for morphine, 3 days for saline) were conducted once daily for 6 days. Immediately after subcutaneous injection (s.c.) injection of morphine (1 mg/kg), animals were placed in one compartment for 1 hour. On alternate days, animals receiving vehicle were placed in the other compartment for 1 hour. On day seven, tests of conditioning were performed as follows. The partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The mice were not treated with either morphine or saline, and then placed on the platform. The time spent in each compartment during a 900-second session was then recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusyo Co., Tokyo, Japan). The preference for drug-paired place was shown as a mean difference between the time spent during the drug-conditioning compartment and saline-conditioning compartment. All sessions were conducted under conditions of dim illumination (28-lux lamp) and white masking noise. In this study, we used 6–16 mice.

Measurement of locomotor activity

The locomotor activity of mice was measured by an ambulometer as described previously (Narita *et al.* 1993). Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the activity cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Total activity counts were automatically recorded for 3 hours following the injection of saline (10 ml/kg, s.c.) or morphine (10 mg/kg, s.c.). In this study, we used 9–10 mice.

[^{35}S]GTPγS binding assay

In the membrane preparation, mice were killed by decapitation and the limbic forebrain was then dissected as described previously (Narita *et al.* 2001). The limbic forebrain was rapidly excised at 4°C, and the tissues were homogenized using a Potter–Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris-Mg^{2+} buffer containing 50 mM Tris-HCl (pH 7.4), $MgCl_2$ and 1 mM ethylene glycol-bis-(beta-aminoethyl ether)-*N,N'*-tetra-acetic acid (EGTA) for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4°C for 10 minutes at 48 000 × g. The pellet was resuspended in [^{35}S]GTPγS binding assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA and 100 mM NaCl and centrifuged at 4°C for 10 minutes at 48 000 × g. The resultant pellet was resuspended in [^{35}S]GTPγS binding assay buffer and stored at −70°C until used. The membrane homogenate (3–8 μg of protein/assay) was incubated at 25°C for 2 hours in 1 ml of assay buffer with various concentrations of dopamine, 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters pre-soaked in 50 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ at 4°C for 2 hours. Filters were then washed three times with 5 ml of ice-cold Tris-HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of

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Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 hours, and the radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

Statistical analysis

Data represent the mean counts with SEM. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

RESULTS

Enhancement of morphine-induced hyperlocomotion in mice exposed to bisphenol-A during the organogenesis or lactation period

Treatment with 10 mg/kg (s.c.) morphine produced a locomotor-enhancing effect in all groups. In both O-2 and L-2 mice, but not I-2 and P-2, the hyperlocomotion induced by morphine was dramatically potentiated as compared with that in control ($P < 0.05$ versus control, Fig. 1).

Enhancement of morphine-induced rewarding effect in mice exposed to bisphenol-A during the organogenesis or lactation period

At the dose of 1 mg/kg, morphine produced neither place preference nor place aversion in control, I-2 and P-2 mice. However, treatment with 1 mg/kg morphine produced a significant place preference in both O-2 and L-2 ($P < 0.05$ versus control, Fig. 2).

The dopamine-induced G-protein activation in the limbic forebrain of mice exposed to bisphenol-A during the organogenesis or lactation period

Dopamine (0.1–10 μM) produced a concentration-dependent increase in [^{35}S]GTPγS binding to membranes from the limbic forebrain including the nucleus accumbens of control, I-2, O-2, P-2 and L-2 mice. It should be noted that the stimulation of [^{35}S]GTPγS binding induced by dopamine was markedly potentiated in O-2 and L-2 mice (Fig. 3).

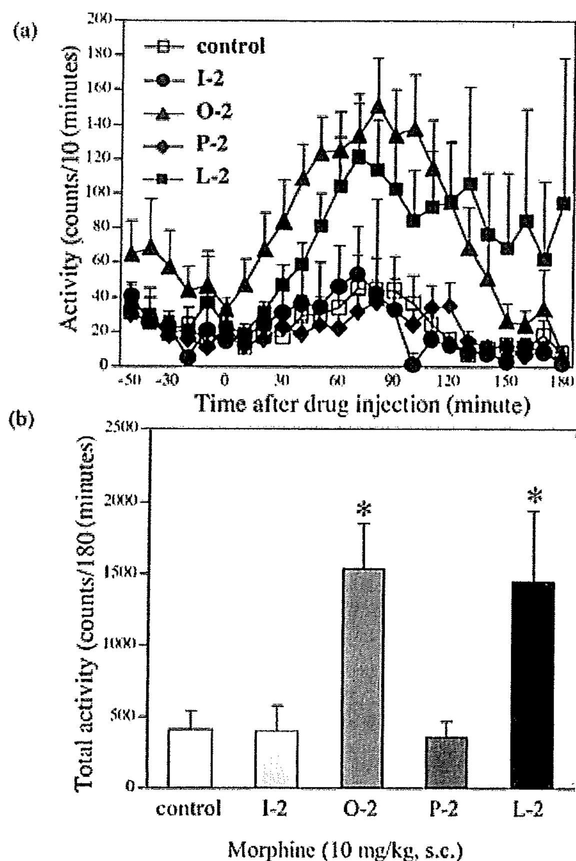

Figure 1 Enhancement of the morphine-induced hyperlocomotion in mice exposed to bisphenol-A during organogenesis or lactation. (a) Time-course changes in the morphine-induced hyperlocomotion in control and bisphenol-A-treated (I-2, O-2, P-2 and L-2) mice (I = implantation, O = organogenesis, P = parturition, L = lactation). Each point represents the mean activity counts for 10 minutes with SEM of 9–10 mice. O-2 (triangle), $F_{(1,299)} = 11.4$, $P < 0.01$ versus control (open square); L-2 (filled square), $F_{(1,299)} = 4.1$, not significant. (b) Total activity in the morphine-induced hyperlocomotion in control and bisphenol-A-treated (I-2, O-2, P-2 and L-2) mice. Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups. Each column represents the mean total activity counts for 180 minutes with SEM of 9–10 mice. *$P < 0.05$ versus control. s.c. = subcutaneous injection

DISCUSSION

Drug addiction in a pathological behavior characterized by compulsive drug seeking and drug ingestion despite severe adverse consequences. The place-conditioning paradigm has become the most frequently used method to evaluate the motivational properties and its use has been reported more frequently than the self-administration paradigm (Suzuki 1996).

Many studies have suggested that the mesolimbic dopaminergic system that projects from the ventral tegmental area (VTA) to the nucleus accumbens is critical for the initiation of opioid reinforcement and hyperlocomotion (Stinus *et al.* 1986; Wise & Rompre 1989; Koob 1992). Either [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO)- or morphine-induced place preference can be blocked by dopamine receptor antagonists (Phillips, LePiane & Fibiger 1983; Shippenberg, Bals-Kubik & Herz 1993). In addition, hyperlocomotion induced by morphine can be blocked by treatment with dopamine receptor antagonists in the nucleus accumbens (Maldonado *et al.* 1990; Funada, Suzuki & Misawa 1994). These

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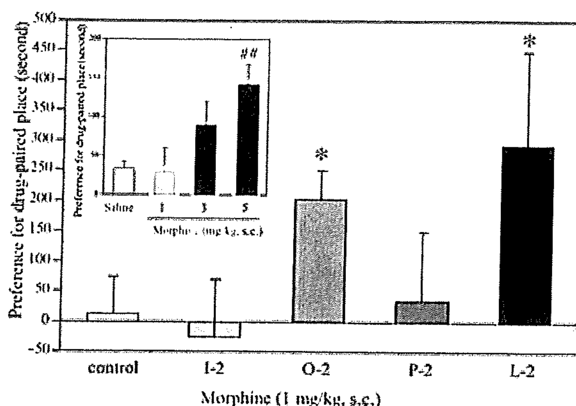

Figure 2 Enhancement of the morphine-induced rewarding effect in mice exposed to bisphenol-A during organogenesis or lactation. (Inner) Dose–response for the morphine-induced place preference in control mice (##$P < 0.01$ versus saline-treated mice). (Outer) Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups (I = implantation, O = organogenesis, P = parturition, L = lactation). Each column represents the mean conditioning score with SEM of 6–16 mice. *$P < 0.05$ versus control. s.c. = subcutaneous injection

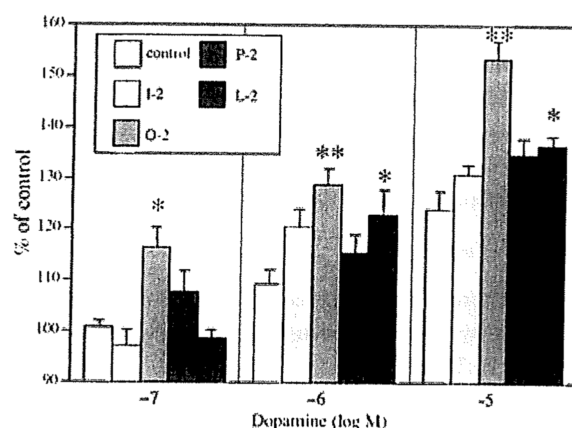

Figure 3 Comparison of the stimulation of [^{35}S]GTPγS binding to membranes from the limbic forebrain by dopamine between control and bisphenol-A-treated mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and guanosine-5′-diphosphate (GDP) (30 μM) with dopamine. The data are shown as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and absence of dopamine. Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups (I = implantation, O = organogenesis, P = parturition, L = lactation). Each column represents the mean with SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus control

findings indicate that the dopamine-containing neuron of the midbrain VTA, which has a high density of μ-opioid receptors, plays a critical role in the rewarding effects and hyperlocomotion by μ-opioid receptor agonists. In terms of dopamine receptor involvement at the terminal site of the mesolimbic dopamine system, the rewarding effects of abused drug have been shown to be mediated by dopamine D_1 receptors (Shippenberg *et al.* 1993; Suzuki *et al.* 1993). In addition, the dopamine D_3 receptor cloned by Sokoloff *et al.* (1990) has been characterized extensively. The dopamine D_3 receptor shows a distinct distribution in limbic areas of the brain, including the nucleus accumbens and olfactory tubercle (Sokoloff *et al.* 1990). Several pharmacological studies with dopamine D_3 receptor-preferring agonists such as 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT) suggest that the dopamine D_3 receptor regulates the inhibitory effect to produce hyperlocomotion in rodents (Suzuki *et al.* 1995; De Boer *et al.* 1997). In addition, we found that the morphine-induced rewarding effect and hyperlocomotion were markedly enhanced in mice lacking the dopamine D_3 receptor gene (Narita *et al.* 2003). These findings suggest that the dopamine D_3 receptor plays a critical role in mediating drug-induced effects on dopamine neurotransmission.

In the previous study, we reported that prenatal and neonatal exposure to bisphenol-A enhances the rewarding effect of drugs of abuse, which is associated with the up-regulation of central dopamine D_1 receptor function and down-regulation of the functional dopamine D_3 receptors in mice (Suzuki *et al.* 2003; Mizuo *et al.* 2004a,b). In the present study, we investigated the relationship between these neuronal changes and the exposure period for bisphenol-A. The exposure to bisphenol-A during either organogenesis or lactation significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. These findings suggest that prenatal and neonatal, especially organogenetical and lactational, exposure to bisphenol-A leads to the supersensitivity of drugs of abuse-induced pharmacological actions.

Morphine has been shown to indirectly activate dopamine neurons in the VTA as a consequence of inhibiting non-dopaminergic neurons, presumably γ-aminobutyric acid-containing neurons, leading to the increased dopamine release in the nucleus accumbens. In a previous study, we reported that prenatal and neonatal exposure to bisphenol-A failed to enhance μ-opioid receptor-mediated G-protein activation by morphine in the lower midbrain (Mizuo *et al.* 2004a). We further showed that the expression of μ-opioid receptor mRNA was not changed by chronic bisphenol-A treatment, suggesting that μ-opioid receptor function is unaffected in this region (Mizuo *et al.* 2004a). Therefore, we next investigated the influence of prenatal and neonatal exposure to bisphenol-A in mesolimbic dopaminergic function using [^{35}S]GTPγS binding assay. The exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These

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results indicate that either organogenesis or lactation is more sensitive to the bisphenol-A-induced neuronal toxicity than any other periods.

Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behaviors (Cagen *et al.* 1999; Farabollini *et al.* 2002). We have already confirmed that acute administration of bisphenol-A with adult mice could not affect the dopamine-related behaviors (data not shown). On the other hand, several investigations clarified the behavioral abnormalities by prenatal and neonatal exposure to bisphenol-A. These findings indicate that prenatal and neonatal exposure to bisphenol-A may cause the neuronal toxicity specifically in the developmental process. In the present study, we focused on the relationship between these developmental changes and the exposure period for bisphenol-A. The exposure to bisphenol-A during either organogenesis or lactation significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. Furthermore, the exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These results indicate that either organogenesis or lactation is more sensitive to the bisphenol-A-induced developmental toxicity than any other periods.

Generally, in cerebral development, it is well known that the proliferation, differentiation or migration of nerve cells and glia cells are carried out most briskly at organogenesis (Temple 2001). Additionally, the functional development of the central nerves system, synaptogenesis and the construction of the nerve network are carried out most briskly at lactation (Temple 2001). Therefore, these reports strongly support our present results that these are most sensitive periods for the influence of bisphenol-A exposure in the development of the central nervous system. Our findings suggest the idea that exposure during organogenesis to bisphenol-A could affect the differentiation or migration of neuronal stem cells. Additionally, lactation exposure to bisphenol-A affects the functional development of the central nervous system including synaptogenesis and the construction of the neuronal network.

Taken together, the present data may explain that although the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behaviors, the prenatal and neonatal exposure, especially either organogenesis or lactation, to bisphenol-A induced developmental neuronal toxicity in the midbrain of rodents. Our findings warn that exposure to bisphenol-A during either organogenesis or lactation may predispose their children to the development of dopamine-related disorders.

Acknowledgements

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〔Original Article〕

Changes in Central Dopaminergic Systems with the Expression of Shh or GDNF in Mice Perinatally Exposed to Bisphenol-A

Kazuya MIYAGAWA*, Minoru NARITA*, Michiko NARITA, Keiichi NIIKURA*, Hisahiko AKAMA*, Yuri TSURUKAWA* and Tsutomu SUZUKI*

* Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences

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Abstract: In the previous study, we reported that exposure to bisphenol-A induced the potentiation of dopamine receptor functions in the mouse limbic area, resulting in supersensitivity to methamphetamine-induced pharmacological actions. The present study was undertaken to investigate whether prenatal exposure to bisphenol-A could produce morphological change in dopaminergic neuron and the pattern of expression of genes regulating the dopaminergic neuron development. Here we found that prenatal and neonatal exposures to bisphenol-A increased the tyrosine hydroxylase- and dopamine transporter-like immunoreactivities in the adult mouse limbic area. The present molecular biological study shows that chronic bisphenol-A treatment produced a significant decrease in the dopaminergic neuron development factors, sonic hedgehog and glial cell line-derived neurotrophic factor, which were also decreased by prenatal exposure to bisphenol-A. These results suggest that chronic exposure to bisphenol-A could disrupt the dopaminergic neurotransmission in the process of dopaminergic neuron development.

Key words: Bisphenol-A, Dopamine, Sonic hedgehog, Glial cell line-derived neurotrophic factor, Endocrine disrupting chemicals

In recent years there has been increasing public concern that chemicals in the environment may affect the endocrine function of humans and wildlife (Colborn, 1995). Bisphenol-A is an environmental endocrine-disrupting chemical that affects reproduction in wildlife. Bisphenol-A is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins, which are used in food cans and found as a contaminant not only in the liquid of the preserved foods, but also in the water autoclaved in the cans (Brotons et al, 1995). This chemical is also released from polycarbonate flasks during autoclaving (Krishnan et al, 1993). Moreover, it has been reported that significant amounts of bisphenol-A are detected in the saliva of dental patients treated with fissure sealants (Olea et al, 1996).

We previously demonstrated that prenatal and neonatal exposures to bisphenol-A markedly enhanced the rewarding effects or hyperlocomotion induced by methamphetamine (Suzuki et al, 2003) and morphine (Mizuo et al, 2004a; Narita et al, 2006). We also demonstrated that in adult mice, prenatal and neonatal exposures to bisphenol-A enhanced function mediated by central dopamine D_1 receptors, which plays a substantial role in the rewarding effect of methamphetamine (Suzuki et al, 2003). These treatments also attenuated the function mediated by the dopamine D_3 receptor subtype that contributes to the inhibitory modulation of postsynaptic dopamine D_1/D_2 receptor-mediated signaling (Mizuo et al, 2004b). These findings indicate that exposure to bisphenol-A during development alters postsynaptic regulation of dopaminergic neurotransmission in the central nervous system (CNS), which results in an enhancement of psychological dependence on drugs of abuse. Although bisphenol-A may affect dopaminergic signaling in the CNS, little is known about the direct role of bisphenol-A in the development of dopaminergic neurotransmission. The purpose of the present study was then to clarify the effect of bisphenol-A on dopaminergic neuron development in mice.

In addition, many recent findings have supported the idea that astrocytes, which are a subpopulation of glial cells, play a critical role in neuronal transmission in the CNS. Their activation may control the structural and functional plasticity of synapses in the CNS. On the other hand, long-term exposure to drugs of abuse can induce neuronal plasticity, and we have shown that treatment of mouse cortical neuron/glia cocultures with methamphetamine or morphine caused morphological changes in astrocytes (Narita et al, 2005). Moreover, treatment with methamphetamine increased the

* Reprint requests should be sent to M. Narita and T. Suzuki
成田 年，鈴木 勉：〒142-8501 東京都品川区荏原 2-4-41
星薬科大学薬品毒性学教室
E-mail: narita@hoshi.ac.jp/suzuki@hoshi.ac.jp

Abbreviations ac: anterior commissure, **ANOVA**: analysis of varience, **BPA**: bisphenol-A, **CNS**: central nervous system, **D_1R**: dopamine D_1 receptor, **D_2R**: dopamine D_2 receptor, **D_3R**: dopamine D_3 receptor, **DAT**: dopamine transporter, **DAMGO**: [D-Ala^2,N-MePhe^4,Gly^5-ol]enkephalin, **EDs**: embryonic days, **FGF**: fibroblast growth factor, **GABA**: γ-aminobutyric acid, **GAPDH**: D-glyceraldehyde-3-phosphate dehydrogenase, **GDNF**: glial cell-line derived neurotrophic factor, **GFAP**: glial fibrillary acidic protein, **LMX**: LIM homeobox transcription factor, **Pax**: paired- and homeodomain-containing transcription factor, **PBS**: phosphate-buffered saline, **RT-PCR**: reverse transcription-polymerase chain reaction, **RXR**: retinoid receptor, **Shh**: sonic hedgehog, **TGF**: transforming growth factor, **TH**: tyrosine hydroxylase, **VTA**: ventral tegmental area

sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (Narita et al, 2005). Furthermore, astrocytes play a critical role in dopaminergic neuron development. We recently reported that *in vitro* treatment of bisphenol-A in mouse-purified astrocytes and neuron/glia cocultures caused the activation of astrocytes, as detected by a stellate morphology and an increase in levels of glial fibrillary acidic protein (GFAP). Therefore, we also investigated here whether prenatal and neonatal exposures to bisphenol-A induces astrocytic activation associated with the alternation of the dopaminergic neuron development.

MATERIALS AND METHODS

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.

Animals

All experiments were performed using 10–14-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 powdered food containing 0 (control) or 2×10^3 μg bisphenol-A/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. In addition, RT-PCR was also performed using embryonic C57BL/6J mice that had been prenatally exposed to bisphenol-A from mating to embryo 14 days (same concentration as mentioned above).

It should be mentioned that the blood level of bisphenol-A in the present study (approximately 10 ng/ml, data not shown) is considered to be more than 30 times higher than the healthy human-exposure level (Inoue et al, 2000). However, our previous study clearly indicate that even much lower concentrations of bisphenol-A exposure (3×10^{-2} μg bisphenol-A/g of food) produced the enhancement of the pharmacological actions induced by morphine (Narita et al, 2006).

RT-PCR

In the RNA preparation and semiquantitative analysis by reverse transcription-PCR, total RNA in the whole brain (adult mice: excluding cerebellum, embryonic mice: including cerebellum) was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the instructions of the manufacturer. First-strand cDNA was prepared as described previously (Narita et al, 2001), and the targeted genes were amplified in 50 μl of a PCR solution containing $MgCl_2$, dNTP mix and DNA polymerase (Invitrogen, Carlsbad, CA) with synthesized primers of dopamine D_1 receptor (103 bp) (sense, 5-CTC ATA AGC TTT TAC ATC CCC G-3; antisense, 5-CCC TCT CCA AAG CTG AGA TG-3), dopamine D_2 receptor (202 bp) (sense, 5-CTC TAC CCT CCA ATC CAC TCC-3; antisense, 5-TAA GGC AGA GGC ACT GGC-3), dopamine D_3 receptor (136 bp) (sense, 5-GCA GTG GTC ATG CCA GTT CAC TAT CAG-3; antisense, 5-CCT GTT GTG TTG AAA CCA AAG AGG AGA GG-3), DAT (540 bp) (sense, 5-AAG ATC TGC CCT GTC CTG AAA G-3; antisense, 5-CAT CGA TCC ACA CAG ATG CCT C-3), Shh (243 bp) (sense, 5-CTG GCC AGA TGT TTT CTG GT-3; antisense, 5-GAT GTC GGG GTT GTA ATT GG-3) or GDNF (403 bp) (sense, 5-ACC AGA TAA ACA AGC GGC AG-3; antisense, 5-TCA GAT ACA TCC ACA CCG TTT AG-3). Samples were heated to 94°C for 5 min, 55°C for 1 min, and 72°C for 1 min, and cycled 35 times through 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. The final incubation was at 72°C for 7 min. The mixture was subjected to 2% agarose gel electrophoresis with the indicated markers and primers for the internal standard glyceraldehyde-3-phosphate dehydrogenase. Each sample was applied to more than two lanes in the same gel. The agarose gel was stained with ethidium bromide and photographed with ultraviolet transillumination. The intensity of the bands was analyzed and quantified by computer-assisted densitometry using NIH Image software.

Immunohistochemistry

In the immunohistochemical approach, mice were deeply anaesthetized with sodium pentobarbital (70 mg/kg, ip) 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 ventral tegmental area (VTA) or the limbic forebrain including the nucleus accumbens were initially dissected using Brain Blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 hr. 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 10% normal horse serum (NHS) in 0.01 M PBS for 1 hr at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10% NGS [1:1000 tyrosine hydroxylase (TH) (Chemicon, Temecula, CA), 1:10 GFAP (NICHIREI, Tokyo, Japan) and 1:2500 DAT (Chemicon)] and incubated for 2 days at 4°C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 for 2 hr 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).

Drugs

The drug used in the present study was bisphenol-A (Wako Pure Chemical Industries Ltd.).

Statistical analysis

All data are expressed as mean ±SEM. Statistical analyses were performed using Student's *t*-test.

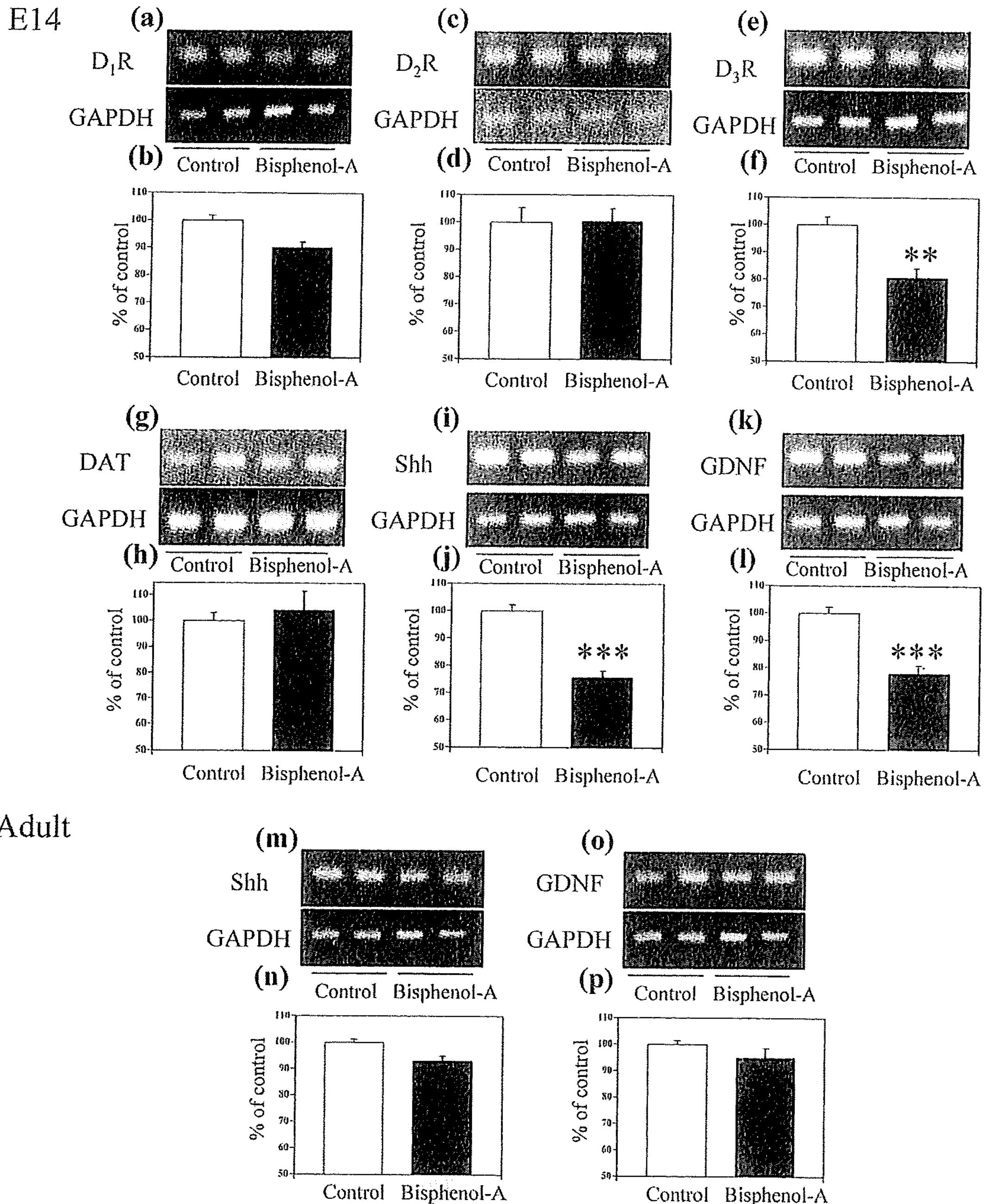

Fig. 1 Down-regulation of the expression of dopamine D_3 receptor, Shh and GDNF mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A. (a, c, e, g, i, k) Representative RT-PCR for the dopamine D_1 receptor (D_1R; a), dopamine D_2 receptor (D_2R; c), dopamine D_3 receptor (D_3R; e), DAT (g), Shh (i) and GDNF (k) mRNAs in the whole brain obtained from embryonic mice. (f, j, l) Significant decrease in the expression of dopamine D_3 receptor (f), Shh (j) and GDNF (l) mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A (filled bar) compared to that from control mice (open bar). (b, d, h) Under these conditions, no changes in mRNA levels of dopamine D_1 receptor (a), dopamine D_2 receptor (d) or DAT (h) were noted. (m, o) Representative RT-PCR for the Shh (m) and GDNF (o) mRNAs in the whole brain minus cerebellum obtained from adult mice. (n, p) No changes in the expression of Shh (n) or GDNF (p) mRNAs in the whole brain minus cerebellum obtained from mice prenatally and neonatally exposed to bisphenol-A (filled bar) as compared to control mice (open bar). The values are expressed as a percentage of the value in the control mice. Each column represents the mean ±SEM of 3 independent samples. **: $P<0.01$, ***: $P<0.001$ vs. control mice.

RESULT

Down-regulation of the expression of dopamine D_3 receptor, Shh and GDNF mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A

In the RT-PCR assay, chronic bisphenol-A treatment produced a significant decrease in the dopamine D_3 receptor ($P<0.01$, Fig. 1e, f), Shh ($P<0.001$, Fig. 1i, j) and GDNF ($P<0.01$, Fig. 1k, l) production in the whole brain obtained from embryonic mice. On the other hand, no changes in mRNA levels of Shh (Fig. 1m, n) or GDNF (Fig. 1o, p) were noted in the whole brain obtained from the adult mice prenatally and neonatally exposed to bisphenol-A. Under these conditions, no changes in mRNA levels of dopamine D_1 receptor (Fig. 1a, b), dopamine D_2 receptor (Fig. 1c, d) and DAT (Fig. 1g, h) were noted in the whole brain obtained from embryonic mice.

Increases in DAT, TH and GFAP-like immunoreactivities in the nucleus accumbens and ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A

We first investigated the possible morphological changes in dopaminergic neurons. The DAT or TH-like immunoreactivity (DAT-IR or TH-IR) in the VTA was observed by immunohistochemical analysis (Fig. 2a–d). Prenatal and neonatal exposures to bisphenol-A failed to induce morphological changes in dopamine cell bodies or the number of dopaminergic neurons. On the other hand, prenatal and neonatal exposures to bisphenol-A produced a dramatic increase in the levels of DAT-IR and TH-IR in the nucleus accumbens (Fig. 2e–h). In addition, GFAP-like immunoreactivity (GFAP-IR) was increased in the mouse ventral pallidum by prenatal and neonatal exposures to bisphenol-A (Fig. 2i, j). Double-labelling experiments showed that the increased DAT-IR was expressed in nonglial cells of the nucleus accumbens obtained from bisphenol-A treated mice, as shown by no apparent colocalization with GFAP-IR (Fig. 2k, l).

DISCUSSION

Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function or social behaviors (Cagen et al, 1999). In the previous study, we found that acute administration of bisphenol-A to adult mice failed to affect the dopamine-related behaviors (Narita et al, 2006). On the other hand, several investigations clarified the behavioral abnormalities from prenatal and neonatal exposures to bisphenol-A (Mizuo et al, 2004a, 2004b; Narita et al, 2006; Suzuki et al, 2003). These findings indicate that prenatal and neonatal exposures to bisphenol-A may cause the neuronal toxicity, specifically in the developmental process. In the previous study, we reported that prenatal and neonatal exposures to bisphenol-A induced the abnormality of the dopamine receptor functions in the mouse limbic area, resulting in supersensitivity of methamphetamine-induced pharmacological actions (Suzuki et al, 2003). These findings indicate that exposure to bisphenol-A during development alters postsynaptic regulation of dopamine neurons. In the present study, we therefore focused on the change in the dopaminergic neuron during development.

Here, we found that chronic bisphenol-A treatment produced a significant decrease in sonic hedgehog (Shh) and glial cell line-derived neurotrophic factor (GDNF) production in the whole brain obtained from embryonic mice. On the other hand, no changes in mRNA levels of Shh and GDNF were noted in the whole brain obtained from the adult mice prenatally and neonatally exposed to bisphenol-A. Progenitor cells develop into dopaminergic neurons through the actions of Shh and fibroblast growth factor 8 (FGF8) (Lee et al, 2000). Maturation is orchestrated by several transcription factors, including the orphan nuclear receptor (Nurr-1), which is widely expressed in both the adult and developing CNS (Zetterstrom et al, 1996). Furthermore, it was reported that Shh, FGF8 and Nurr-1 collaborate to induce dopaminergic phenotypes (Kim et al, 2003). GDNF is also one of the most potent trophic factors for dopaminergic neurons, playing a role in development and survival (Lin et al, 1993). Therefore, the present data support the idea that prenatal and neonatal exposures to bisphenol-A may disrupt dopaminergic neuron development associated with the expression of Shh and GDNF.

We previously reported that prenatal and neonatal exposures to bisphenol-A induced the functional reduction in dopamine D_3 receptors in mice (Mizuo et al, 2004b). Du et al. reported that the pharmacological action of GDNF was regulated by activation of dopamine D_3 receptor (Du et al, 2005). In our previous study, exposure to bisphenol-A during organogenesis (Embryonic days (EDs) 7–14), but not implantation (EDs 0–7) or parturition (EDs 14–20), significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. Furthermore, exposure to bisphenol-A during organogenesis also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These findings strongly support our hypothesis that bisphenol-A disrupts dopaminergic neuron development.

Next, we further investigated whether prenatal and neonatal exposures to bisphenol-A could affect the dopaminergic neuron in the adult brain. Immunohistochemical study showed that prenatal and neonatal exposures to bisphenol-A failed to change DAT-IR and TH-IR in the VTA. These results suggest that prenatal and neonatal exposures to bisphenol-A failed to induce cell death, overexpression of or morphological changes in dopaminergic neuron in the VTA. On the other hand, we found that prenatal and neonatal exposures to bisphneol-A dramatically increased DAT-IR and TH-IR in the nucleus accumbens. These results suggest that prenatal and neonatal exposures to bisphenol-A induce the abnormalities at axon terminals of dopaminergic neurons.

Another key finding of the present study was that prenatal and neonatal exposures to bisphenol-A induced astroglial proliferation as characterized by the increase in GFAP-IR levels, and astroglial hypertrophy as detected by a stellate morphology of GFAP-IR in the ventral pallidum. Many toxic stimuli activate astrocytes. The activation of astrocytes may control the structural and functional plasticity of synapses in the

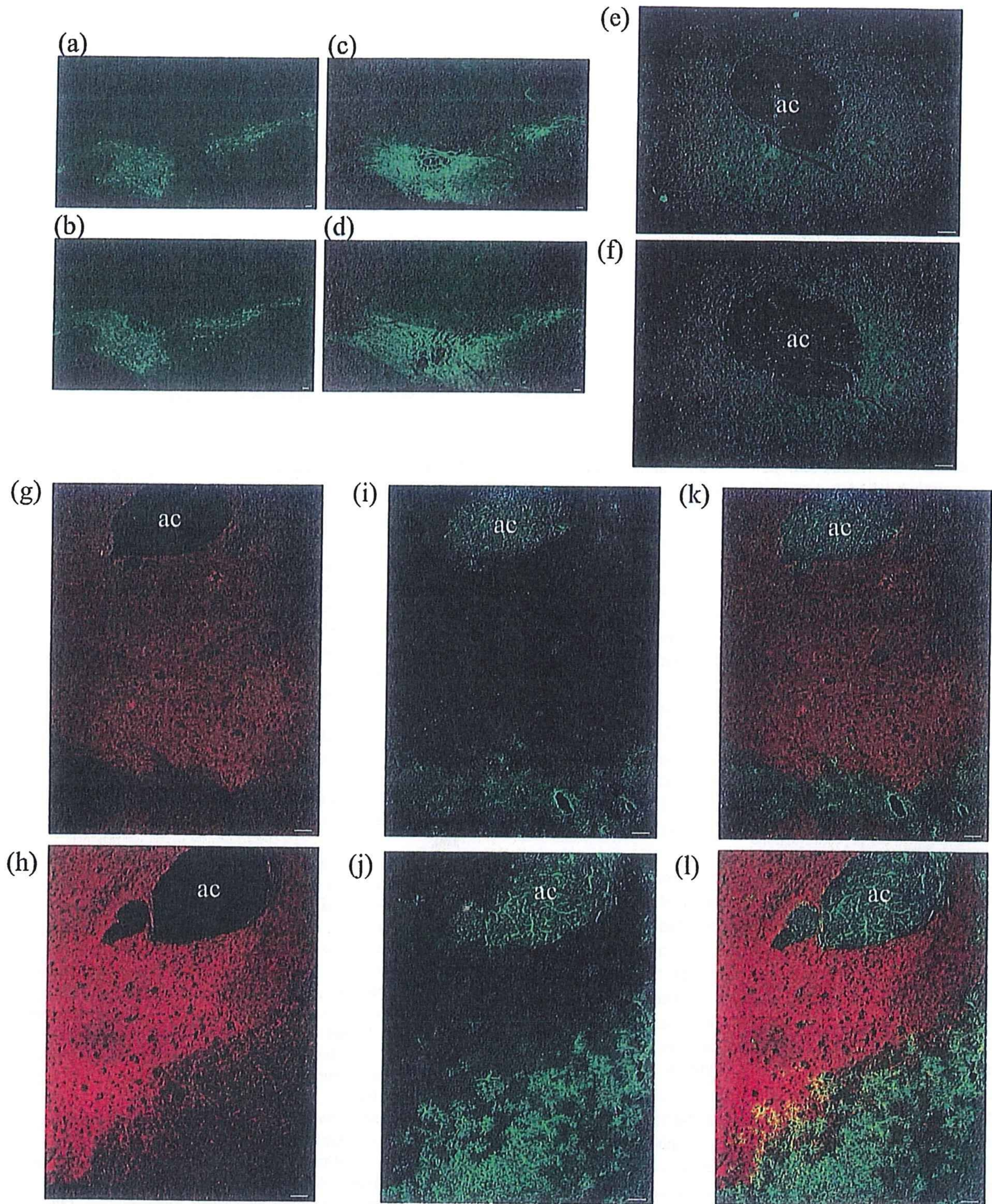

Fig. 2 Increase in DAT-, TH- and GFAP-IRs in the nucleus accumbens or ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A. (a, b) The TH-IR in the ventral tegmental area (VTA) did not change in mice prenatally and neonatally exposed to bisphenol-A (b) compared to control mice (a). (c, d) Similarly, no change of the DAT-IR in the VTA was noted in mice prenatally and neonatally exposed to bisphenol-A (d) compared to control mice (c). (e, f) On the other hand, the increased TH-IR in the nucleus accumbens was noted in mice prenatally and neonatally exposed to bisphenol-A (f) compared to control mice (e). (g, h) The increased DAT-IR in the nucleus accumbens was also observed in mice prenatally and neonatally exposed to bisphenol-A (h) compared to control mouse (g). The GFAP-IR in the ventral pallidum was dramatically increased with morphological changes in mice prenatally and neonatally exposed to bisphenol-A (j) compared to control mice (i). The green labeling for DAT and the red labeling for GFAP show no apparent colocalization in the limbic area (k, l). ac: anterior commissure. Scale bars: 50 μm.

CNS. Recent accumulating evidence suggests that astrocytes express a repertoire of neurotransmitter receptors mirroring that of neighboring synapses. Such receptors are stimulated during synaptic activity and spread information by calcium signaling into the astrocyte network via gap-junction channels (Pasti et al, 1997). It has been widely accepted that long-term exposure to drugs of abuse can induce neuronal plasticity. We have shown that treatment of mouse cortical neuron/glia cocultures with methamphetamine or morphine causes morphological changes in astrocytes (Narita et al, 2005). Moreover, treatment with methamphetamine increases the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (Narita et al, 2005). Interestingly, treatment of mouse purified astrocytes and neuron/glia cocultures with bisphenol-A caused the activation of astrocytes, as detected by stellate morphology and an increase in levels of GFAP (Miyatake et al, 2006). It has been reported that the projection from the nucleus accumbens to the ventral pallidum regulates the reinstatement of cocaine seeking behavior in rats extinguished from cocaine self-administration (Tang et al, 2005). The nucleus accumbens and ventral pallidum have a pivotal role in regulating exploratory motor behaviors. Pharmacological manipulation of dopamine or enkephalin transmission in the nucleus accumbens induces motor activity. The nucleus accumbens has a prominent GABAergic projection to the ventral pallidum. The motor response elicited by microinjecting the μ-opioid agonist D-Ala-Tyr-Gly-NMePhe-Gly-OH (DAMGO) or dopamine into the accumbens is blocked by stimulating $GABA_A$ receptors in the ventral pallidum with the agonist muscimol (Austin and Kalivas, 1989). Collectively, these reports strongly support the idea that the astrocytic activation in the ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A plays a critical role in the supersensitivity to methamphetamine following bisphenol-A treatment.

As mentioned above, prenatal and neonatal exposures to bisphenol-A may dramatically change the dopaminergic transmission. Knaak and Sullivan first reported the metabolic fate of bisphenol-A in rats (Knaak and Sullivan, 1966), 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. Many reports have showed that bisphenol-A is metabolized and excreted rapidly (Volkel et al, 2002). In addition, we reported that acute administration of bisphenol-A to adult mice did not affect the dopamine-related behaviors (Narita et al, 2006). In our preliminary biochemical studies, bisphenol-A did not increase or decrease [^{35}S]GTPγS bindings to brain membranes. In addition, we could not make the Scatchard plot using [^{3}H] bisphenol-A in brain membranes. Taken together, it is almost impossible that bisphenol-A that remains in the adult brain of mice directly affects dopaminergic neurotransmission.

At the present time, it is very hard to anticipate where the primary site of bisphenol-A is. Although bisphenol-A has weak estrogenic activity, prenatal and neonatal exposures to 17β-estradiol failed to induce supersensitivity to morphine (Miyatake et al, 2006). In addition, treatment with 17β-estradiol failed to induce astrocytic activation (Miyatake et al, 2006). Furthermore, although it is well known that bisphenol-A disrupts thyroid hormone, prenatal and neonatal exposures to propylthiouracil, a thyroid hormone inhibitor, reduced the activation of dopaminergic neurons (unpublished observation). These findings indicate that disruption of dopaminergic neuron development induced by prenatal and neonatal exposures to bisphenol-A can be mediated by non-hormonergic actions of bisphenol-A.

In conclusion, the present data suggest that bisphenol-A induces dopaminergic amplification following the disruption of the dopaminergic neuron development. This phenomenon could explain the aggravation of the development of dependence on drugs of abuse.

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Minireview

Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor

Kaname Kawajiri [a,b], Yoshiaki Fujii-Kuriyama [b,c,*]

[a] *Research Institute for Clinical Oncology, Saitama Cancer Center, Ina-machi 818, Kitaadachi-gun, Saitama 362-0806, Japan*
[b] *Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, Honcho 4-1-8, Kawaguchi 332-0012, Japan*
[c] *Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba 303-8577, Japan*

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that functions as an intracellular mediator in the xenobiotic signaling pathway. Although a number of studies have examined AhR-mediated *CYP1A1* induction in detail, recent studies of AhR-null mice have revealed that AhR plays important regulatory roles in the normal homeostasis and development of animals. In this short review, we summarize the present state of knowledge about the molecular mechanisms of AhR-mediated CYP1 induction, and we also focus on recent advances in the study of the physiological functions of AhR.
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Keywords: Cytochrome P450; CYP1A1; AhR; TCDD; Gene regulation; Nuclear-cytoplasmic transport; Carcinogenesis; Reproduction; Inflammation

Members of the cytochrome P450 (CYP)[1] superfamily of hemoproteins are found across a wide range of species from bacteria to vertebrates [1]. These iron-containing proteins catalyze the monooxygene reaction of various endogenous and exogenous substrates and are classified according to the similarity of their amino-acid sequences. There are 18 CYP mammalian gene families, and four of these families (1, 2, 3, and 4) mainly metabolize foreign compounds including drugs, food additives and environmental pollutants [2]. Members of these CYP families are often specifically induced in response to exposure to foreign chemicals [3].

The molecular mechanisms regulating the induction of CYP proteins have been extensively examined, and a number of different inducers, *cis*-acting DNA elements, *trans*-acting transcription factors, and coactivators have been identified, and these studies have been greatly facilitated by the relatively recent development of novel genetic techniques, such as gene-knockouts, chromatin immunoprecipitation (Chip) and small interfering RNA (siRNA). The induction of CYP1 family member expression is regulated by a heterodimer composed of the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator (Arnt), which contains a characteristic basic-helix–loop–helix (bHLH) and PER-Arnt-SIM (PAS) homology region [4,5]. In contrast, the expression of CYP2, 3, and 4 family members is regulated by the nuclear receptors CAR, PXR (SXR), and PPAR, respectively, all of which possess a nuclear receptor gene family (family NR1) zinc finger motif and form heterodimers with the

* Corresponding author. Address: Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba 303-8577, Japan. Fax: +81 29 853 7318.
E-mail address: ykfujii@tara.tsukuba.ac.jp (Y. Fujii-Kuriyama).

[1] *Abbreviations used:* AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; Chip, chromatin immunoprecipitation; siRNA, small interfering RNA; Arnt, aryl hydrocarbon receptor nuclear translocator; bHLH, basic-helix–loop–helix; PAS, PER-Arnt-SIM; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; NLS, nuclear localization signal; NES, nuclear export signals; RXR, retinoid X-receptor; XRE, xenobiotic responsive element; BTE, basic transcription element; AhRR, AhR repressor; LOH, loss of heterozygosity; PAHs, polycyclic hydrocarbons; CA-AhR, constitutively active form of AhR; SOCS-2, suppressor of cytokine signaling 2.

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retinoid X-receptor (RXR) [3]. In this short review, we summarize our present understanding of the molecular mechanisms controlling the expression of CYP1 family members mediated by AhR with an emphasis on recent studies examining the physiological function(s) of AhR. The mechanisms of NR1-induced expression of CYP2, 3, and 4 family members have been addressed in a number of excellent recent review articles [6–8] , and interested readers should refer to those works.

Molecular mechanisms of CYP1 induction mediated by AhR

Activation of AhR

It is well established that ligation and activation of AhR by endogenous or exogenous compounds such as 3-methylcholanthrene (MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) leads to nuclear transport and transcriptional activation. AhR contains a nuclear localization signal (NLS) in its bHLH region [9], and mice harboring a mutation in the conserved NLS are resistant to TCDD-induced toxicity [10], consistent with results seen in AhR-deficient mice. AhR contains nuclear export signals (NES) in both the bHLH [9] and PAS domains [11] and can shuttle between the cytoplasm and the nucleus. This process is inhibited by LMB, a specific inhibitor of CRM1-dependent nuclear export [12].

In the absence of ligands, AhR is associated with a cytoplasmic protein complex consisting of Hsp90, p23, and ARA9 (XAP2 or AIP). Hsp90 binding is thought to mask the AhR–NLS, and this interaction is essential for the cytoplasmic retention of AhR [13]. Overexpression of ARA9 increases the fraction of AhR found in the cytosol [14], and the LxxLL motif of the NR box in AhR, which was found to mediate protein–protein interactions of transcriptional cofactors with nuclear receptors, is also involved in the cytoplasmic retention of AhR by protein–protein interactions [15]. Additionally, protein kinase C-mediated NLS phosphorylation inhibits the ligand-dependent nuclear import of AhR. Taken together, these findings suggest that the regulation of AhR nuclear import has two distinct components: ligand binding initiates the interaction of the NLS with nuclear import components, and this is negatively regulated by NLS phosphorylation [16] (Fig. 1b).

Several studies have examined the ligand-independent activation of AhR. When several different cell lines, such as Hepa-1 and 10T1/2 fibroblast clonal sublines, are grown in suspension culture, AhR nuclear translocation and the induction of *CYP1A1* or *CYP1B1* mRNA occur in the absence of xenobiotic AhR ligands [17,18]. Additionally, preventing cell–cell interactions of the keratinocyte cell line HaCaT by growing at a low cell density or in Ca^{2+}-deficient S-MEM induces the nuclear accumulation of AhR and promotes the expression of a reporter gene driven by the xenobiotic responsive element (XRE) sequence [19]. In these cells, *Slug*, a member of the snail/slug family of zinc finger transcriptional repressors critical for epithelial–mesenchymal transitions, is transcriptionally activated following AhR nuclear translocation [20]. Furthermore, the second messenger cAMP, an endogenous mediator of hormone and neurotransmitter signaling, is also reported to activate AhR and lead to its nuclear translocation [21]. Finally, omeprazole, a benzimidazole derivative used clinically as an inhibitor of the gastric proton pump, induces CYP1A1 expression in an AhR-dependent manner, but it does not directly bind AhR [22,23]. Ligand-independent AhR activation suggests cross-talk between AhR and other signaling pathways, but further studies are needed to clarify the mechanisms regulating this pathway.

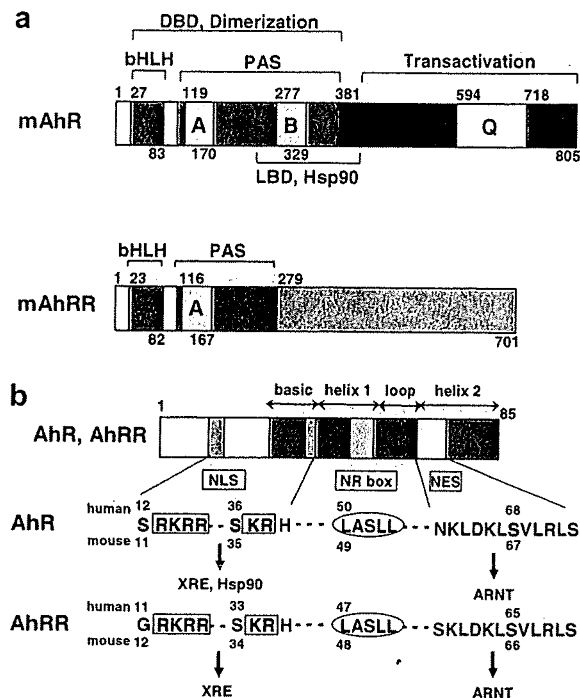

Fig. 1. (a) Schematic representation of functional domain of AhR and AhRR. A, B: weakly homologous repeated regions. Q: glutamine-rich transcription activation region. LBD, Hsp90 ligand binding and Hsp90 interaction domain. DBD DNA binding domain. (b) Schematic representation of the NH_2-terminal functional domains of AhR and AhRR in relation to signals for nuclear import or export. It is noted that a ligand-dependent nuclear import of AhR is inhibited by substitution of aspartic acid for Ser-12 or Ser-36 in human AhR [16], which mimics the negative charge of phosphorylation. On the other hand, a nuclear export of AhR is inhibited by substitution of Ser-68 in human AhR [19].

Transcriptional regulation of CYP1 genes by AhR

The TCDD-induced expression of *CYP1A1* is mediated through the XRE [24]. The core consensus sequence of XRE is 5′-TNGCGTG-3′, and this site is recognized by the AhR/Arnt heterodimer. Approximately 1 kb upstream of the *CYP1A1* gene, a cluster of XREs functions as an enhancer element, and a basic transcription element (BTE), a GC box sequence localized to the proximal