chronic BPA treatment, and this potentiation was reversed by the blockade of dopamine D1 receptors. These findings provide direct evidence for the sustained activation of the dopamine D1 receptor to modulate its signal transduction following prenatal and neonatal exposure to BPA.

The dopamine transporter (DAT: also designed the dopamine reuptake site) has been recognized as a major target for psychostimulants to regulate changes in synaptic dopamine levels and turnover (Giros and Caron, 1993; Fumagalli et al., 1998). By contrast, the type 2 vesicular monoamine transporter (VMAT2) is known to transport monoamine from the cytoplasm into secretory vesicles (Gonzalez et al., 1994). It has been recently proposed that measurements of VMAT2 may provide estimates of monoaminergic neuron terminal numbers (Nirenberg et al., 1996; Wang et al., 1997). In the present Western blotting assay, no changes in protein levels of either DAT or VMAT2 in the whole brain were noted by chronic BPA treatment. These findings indicate the lack of change in levels of proteins to presynaptically modulate the dopaminergic transmission in this event.

BPA and alkylphenols have been reported to have estrogenic activity (Lutz and Kloas, 1999; Kloas et al., 1999; Gaido et al., 1997). Recent molecular studies have suggested the transcriptional activation of the human D1 dopamine receptor gene by estrogen (Lee and Mouradian, 1999). Although further clarification of this molecular mechanism is needed, it is therefore likely that the up-regulation of dopamine D1 receptor following prenatal and neonatal exposure to BPA may lead to the supersensitivity of methamphetamine-induced pharmacological actions.

Public attention and research efforts are being driven by an understanding of the ever-increasing problems and magnitude of substance abuse. In the present study, we demonstrated for the first time that several pharmacological actions of methamphetamine related to psychological dependence are clearly enhanced by prenatal and neonatal exposure to BPA. Our findings warn that chronic exposure to BPA in females may predispose their children to the craving for and relapse of psychostimulants.

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Non-genomic modulation of dopamine release by bisphenol-A in PC12 cells

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Abstract

An endocrine disruptor chemical, bisphenol-A (BPA), is reported to have several short-term actions in various tissues and/or cells; however, the mechanisms of these actions have not been fully elucidated. We investigated short-term actions evoked by BPA in pheochromocytoma PC12 cells. BPA elicited dopamine release in PC12 cells in a dose-dependent manner. A selective N-type calcium channel antagonist (ω-conotoxin GVIA) and a ryanodine receptor blocker (ryanodine) inhibited the BPA-induced dopamine release. The expression of ryanodine receptor mRNA was detected by RT–PCR in PC12 cells. Subsequently, in order to prove whether membrane receptors participate in BPA-evoked dopamine release, a guanine nucleotide-binding protein inhibitor [guanosine 5′-(β-thio) diphosphate], cyclic AMP antagonist (Rp-cAMPS) or protein kinase A inhibitor (H7 or H89) was added to PC12 cells prior to BPA-treatment. All of these agents suppressed BPA-evoked dopamine release, indicating that multiple signaling pathways may be involved in BPA-evoked dopamine release in PC12 cells. In conclusion, we demonstrated that BPA induced dopamine release in a non-genomic manner through guanine nucleotide-binding protein and N-type calcium channels. These findings illustrate a novel function of BPA and suggest that exposure to BPA influences the function of dopaminergic neurons.

Keywords: bisphenol-A, non-genomic actions, dopamine release, guanine nucleotide binding protein, cyclic AMP, N-type calcium channels.

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Bisphenol-A [2,2-Bis(4-hydroxyphenyl)-propane (BPA)] is commonly ingested by humans, being released from polycarbonate plastics (Krishnan *et al.* 1993), the lining of food cans (Brotons *et al.* 1995) and dental sealants (Olea *et al.* 1996). BPA has structural homology with a ring of β-estradiol and is suspected to bind to nuclear estrogen receptors, to mimic genomic actions of estrogen, and to have adverse effects on reproductive function in humans and wild animals (Stone 1994). For example, it was demonstrated that BPA increased prostate size and decreased epididymal weight (Gupta 2000), and influenced proliferative activity in the epithelial cells of the mammary gland (Colerangle and Roy 1997). Furthermore, perinatal exposure to BPA affects behavioral patterns, namely, it causes the masculinization of female behavior (Dessi-Fulgheri *et al.* 2002) and the changes in maternal behavior in mice (Palanza *et al.* 2002). In addition, our previous studies revealed that pre-natal exposure to BPA led to a reduction of dopamine content in the brains of mice (Hiroi *et al.* 2002); however, the mechanism of this effect has not been defined.

Recent studies demonstrated that BPA had some non-genomic actions in various tissues and/or cells through membrane receptors or membrane channels. BPA rapidly activated cyclic AMP responsive element-binding protein (CREB) in pancreatic β cells (Quesada *et al.* 2002), BPA also stimulated nitric oxide synthesis in mouse endothelial

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Abbreviations used: ω-AgTX, ω-Agatoxin IVA; BPA, bisphenol-A or 2,2-Bis(4-hydroxyphenyl)-propane; 17β-estradiol, 1,3,5(10)-estratriene-3,17β-diol; Ca^{2+}, calcium; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; cDNA, complementary DNA; ω-CgTX, ω-conotoxin GVIA; CICR, calcium-induced calcium release; DMSO, dimethyl sulfoxide; GDPβs, guanosine 5′-(β-thio)diphosphate; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; G, protein, guanine nucleotide binding protein; HPLC-ECD, high performance liquid chromatography coupled with electrochemical detection; MAO, monoamine oxidase; mRNA, messemger RNA; PCB, polychlorinated biphenyl; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TH, tyrosine hydroxylase.

cells (Noguchi *et al.* 2002) and, although low concentrations of BPA potentiated $GABA_A$ receptor responses, high concentrations inhibited them (Aoshima *et al.* 2001). However, non-genomic effects of BPA on dopaminergic neurons remain unclear.

PC12 cells are a continuous cell line derived from a rat adrenal gland tumor that synthesize, store, release, and metabolize dopamine and norepinephrine in a manner analogous to that observed *in vivo* (Greene and Tischler 1976; Greene 1977). Thus, PC12 cells are useful models to investigate dopaminergic functions. Extended exposure of PC12 cells to polychlorinated biphenyls (PCBs), endocrine disruptors, has been reported to decrease the cellular content of dopamine. This mechanism appears to be mainly due to an alteration in the synthesis of dopamine. On the other hand, short-term exposure to PCBs for less than an hour increased cellular dopamine content to inhibit the spontaneous release of dopamine (Seegal *et al.* 1989).

In this study, we examined the non-genomic effect of BPA on dopaminergic cells using PC12 cells as a model. BPA has structural homology with β-estradiol (Stone 1994) and the genomic response by β-estradiol requires at least 30 min (Orimo *et al.* 1993). To exclude the genomic influences, short-term exposure (10 min) to BPA was adopted. We found that following treatment with BPA, PC12 cells rapidly released dopamine and the process likely involved several components of signal transduction such as guanine nucleotide binding protein (G protein) and N-type calcium (Ca^{2+}) channels.

Experimental procedures

Materials

Diltiazem, ω-agatoxin-IVA (ω-AgTx), GDPβs, Rp-cAMPS, H-7, H89, ruthenium red, ryanodine, pargyline, clorgyline and RPMI-1640 growth medium were purchased from Sigma (St Louis, MO, USA). ω-Conotoxin GVIA (CgTx) was purchased from Alomone Laboratories (Jerusalem, Israel). Bisphenol-A and all other chemicals unless otherwise noted were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Pheochromocytoma cell culture and BPA exposure

PC12 cells were obtained from the Health Science Research Resources Bank (Cell number JCRB 0733, Osaka, Japan). PC12 cells were cultured in RPM-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin and incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells (1×10^6) were seeded on 100-mm-diameter plastic dishes and used for experiments 1 day later.

BPA was dissolved in dimethyl sulfoxide (DMSO) and diluted into the cell culture medium to achieve the desired concentration. Cells exposed only to DMSO were used as controls. The final DMSO concentrations in the cell culture media were at most 0.1% (v/v). The PC12 cells were washed three times, and incubated at 37°C for 40 min with a low potassium-containing buffer consisting of 140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$ $2H_2O$, 1.2 mM $MgSO_4$ $7H_2O$, 11 mM glucose, and 15 mM HEPES at pH 7.4. Then, various concentrations of BPA were added to the PC12 cells for 10 min at 37°C. If necessary, a Ca^{2+} channel inhibitor, a ryanodine receptor blocker, a G protein antagonist, or a cyclic AMP/PKA antagonist was added for 30–40 min prior to BPA. The buffer was gathered and used to quantitate the release of dopamine. PC12 cells were rinsed three times with phosphate-buffered saline and collected by centrifugation at 1000 *g* for 5 min. Subsequently, the cells were disrupted by ultrasonication with 0.1 mL of 0.2 mM perchloric acid, transferred to microcentrifuge tubes and centrifuged at 120 000 *g* for 15 min at 4°C to separate the cellular components. The supernatants were filtrated with a 0.22-μm filter prior to injection for high-performance liquid chromatography (HPLC) and used for the measurements of intracellular dopamine content.

Measurement of dopamine with HPLC

The amounts of dopamine in cell extracts and released into buffers were measured by HPLC coupled with electrochemical detection (HPLC-ECD; Seegal *et al.* 1986). The HPLC-ECD system consisted of a pump (model LC-9 A, Shimadzu, Japan), sample injector (model AS-8000, TOSOH, Japan), C18 column 5 μM 150 × 4.6 mm (MC Medical Inc., Tokyo, Japan), and an electrochemical detector (model Coulochem II, ESA, Chelmsford, MA, USA). The mobile phase was as follows: 50 mM Na_2HPO_4 $12H_2O$, 50 mM citric acid, 0.1 mM disodium EDTA, 4.4 mM 1-heptanesulfonic acid sodium salt monohydrate, 8.58% methanol, and 3.52% acetonitrile adjusted to pH 3.0. The working electrode potential was set at + 0.4 V versus an Ag-AgCl reference electrode. Routinely, 100 μL of sample was injected onto the column at a rate of 0.9 mL/min. The amount of dopamine in the samples was estimated from the area of the dopamine peak on the chromatogram.

RT–PCR analyses of ryanodine receptor isoforms

Total RNA was extracted from PC12 cells with an Isogen kit (Nippon Gene, Tokyo, Japan) and reverse-transcribed using RNA PCR kit (AMV) Ver. 2.1 (TaKaRa Biologicals, Shiga, Japan) according to the manufacturer's instructions. In brief, for conversion of total RNA to complementary DNA (cDNA), a 20-μL reaction mixture was prepared containing 0.25 units of reverse transcriptase, 1 × RNA–PCR buffer (10 mM Tris–HCl, pH 8.3–50 mM KCl), 1 mM dNTPs, 2.5 μM random 9 mers primer, 0.5 units RNase inhibitor, 5 mM $MgCl_2$, and 2 μg of total RNA. The reaction was carried out at 55°C for 60 min. Reverse transcription was terminated by heating to 99°C for 5 min, then quick chilling on ice. Reverse transcriptase reaction mixtures, including cDNA products, were stored at −20°C until used. A single cDNA produced from total RNA was amplified by PCR using ampli*Taq* Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) with primers specific for ryanodine receptor isoforms, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. For the PCR amplification of cDNA, 50 μL of reaction mixture was prepared containing 1 × PCR buffer (10 mM Tris–HCl, pH 8.3–50 mM KCl), 1.5 mM $MgCl_2$, 200 μM dNTPs, 1.25 units of ampli*Taq* Gold DNA polymerase, an aliquot (1 μL) of the cDNA products (20 μL) from

Table 1 PCR primers used for amplification of ryanodine receptor cDNAs

Isoform of ryanodine receptor	Forward primer	Reverse primer	Size (bp)
Type 1	F01 5'-CCTCCTGGGCCACTACAATA'3'	R01 5'-CTTGCGGAAGAAGTTGAAGG-3'	187
	F02 5'-GAAAGCAGCTGGTGATGACA-3'	R02 5'-GACGACCCGGTACAGTTCAT-3'	245
Type 2	F01 5'-CCAACATGCCAGACCCTACT-3'	R01 5'-GAGACCAGCATTTGGGTTGT-3'	212
	F02 5'-GCCATCAATTTCATCCTGCT-3'	R02 5'-CCACTGGCCTTTGATATCGT-3'	360
Type 3	F01 5'-CCTGGGCCACTACAACAACT-3'	R01 5'-TCATATCCGGCTCATCATCA-3'	224
	F02 5'-CGTCTCTGGTGTCATGGCTA-3'	R02 Same as Type 3 R01	339

The design of the PCR primers was based on the published partial sequences of rat ryanodine receptors (type 1: GeneBank accession no. AF130879, type 2: no. U95157, type 3: no. AF130881). Listed are the two sets of forward (F01–F02) and reverse primers (R01–R02) that target the type 1, 2, or 3 ryanodine receptor isoforms. The predicted sizes (in base pairs) of the PCR products are listed on the right.

the reverse transcriptase reaction, and a 200-nM concentration of the upstream and downstream specific primers listed in Table 1. The thermal cycling consisted of an initial denaturation step for 5 min at 95°C, followed by 36 cycles of: 30 s at 95°C, 60 s at 55°C, and 90 s at 72°C. The final extension step was for 5 min at 72°C. The PCR products were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining under UV light. Images of the gels were taken with a Color Image Freezer (Atto Corp., Tokyo, Japan).

Data analysis

All data are expressed as means ± SEM values. Statistical analysis of the data was performed by unpaired Student's *t*-test or one-way analysis of variance (ANOVA). A Dunnett's post-hoc test for multiple comparisons of paired data was carried out when significant differences were detected by ANOVA. In all HPLC data except for Fig. 1(a), statistical analysis was conducted using the raw data, then converted to percent control. Differences were considered significant when probability values were < 0.05.

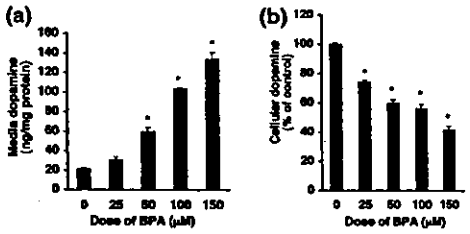

Fig. 1 Effect of BPA-exposure on PC12 cells. (a) Effect of BPA on the concentration of dopamine in the medium. PC12 cells were exposed to various concentrations of BPA for 10 min and the dopamine concentration was measured by HPLC-ECD as described in Experimental procedures. (b) Effect of BPA on intracellular dopamine content. PC12 cells were exposed to various concentrations of BPA for 10 min and intracellular dopamine content was measured by HPLC-ECD as described in Experimental procedures. Each intracellular dopamine content was expressed as a percentage of the control with only vehicle. Values are means ± S.E.M. from three different experiments. *$p < 0.05$, compared with the control containing only vehicle by ANOVA and posthoc Dunnett's.

Results

BPA-induced dopamine release in PC12 cells

To examine the effects of BPA on the intracellular dopamine content and the secretion of dopamine, PC12 cells were treated with various concentrations (25–150 μM) of BPA. In order to exclude the genomic effect of dopamine, a short-term BPA-treatment (10 min) was adopted. The exposure to BPA for 10 min increased the media dopamine concentration in a dose-dependent manner (Fig. 1a) while decreasing the intracellular dopamine content (Fig. 1b). The control value in Fig. 1(b) was 333.7 ± 3.2 ng/mg protein. This suggests most likely that BPA elicited the release of dopamine.

BPA-induced intracellular dopamine reduction in PC12 cells is independent of metabolic influences

In PC12 cells, there are three metabolic pathways capable of converting dopamine to its metabolites. The primary pathway is catalyzed by monoamine oxidase (MAO; Greene and Rein 1978; Tuler *et al.* 1989). To investigate the influence of this metabolic pathway, 1 μM of clorgyline, a selective inhibitor of MAO type A, or 100 μM of pargyline, an inhibitor of both MAO type A and B, was added for 30 min prior to 50 μM BPA. The concentrations of clorgyline and pargyline used in this study were sufficient to inhibit MAO (Basma *et al.* 1990; Li *et al.* 1992). Single exposure to BPA reduced the intracellular dopamine content to 271.4 ± 3.4 ng/mg protein (control: 336.0 ± 1.7 ng/mg protein). Treatment with both clorgyline and BPA decreased the intracellular dopamine content to 265.4 ± 5.9 ng/mg protein (treatment with clorgyline: 324.6 ± 4.2 ng/mg protein). Treatment with both pargyline and BPA also reduced the intracellular dopamine content to 270.2 ± 2.4 ng/mg protein (treatment with pargyline: 340.1 ± 3.8 ng/mg protein). The intracellular dopamine content under these conditions did not vary significantly, suggesting that neither 1 μM clorgyline nor 100 μM pargyline was capable of preventing the reduction in intracellular dopamine that occurred in PC 12 cells upon exposure to 50 μM BPA for 10 min (Fig. 2). Two minor pathways of dopamine metabolism in PC12 cells involve the formation of

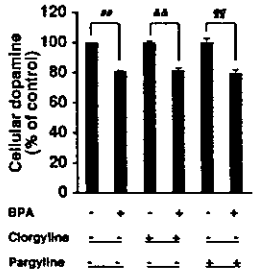

Fig. 2 Effect of MAO inhibitor on BPA-evoked intracellular dopamine decrease. PC12 cells were treated with either 1 μM clorgyline or 100 μM pargyline for 30 min prior to 50 μM BPA and intracellular dopamine content was measured by HPLC-ECD as described in Experimental procedures. Each intracellular dopamine content is expressed as a percentage of the control containing only vehicle. Values are means ± SEM from three different experiments. ##$p < 0.01$, compared with the control containing only vehicle. &&$p < 0.01$, compared with the control containing only 1 μM clorgyline. ¶¶$p < 0.01$, compared with the control containing only 100 μM pargyline. Each statistical analysis was performed by unpaired Student's *t*-test.

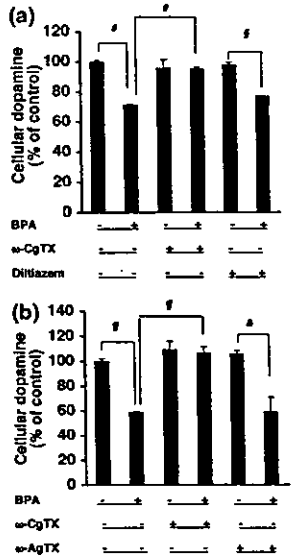

Fig. 3 Effect of pre-treatment with each selective Ca^{2+} channel blocker on BPA-evoked release of dopamine. (a) Effect of pretreatment with ω-CgTX, a N-type Ca^{2+} channel blocker, or diltiazem, a L-type Ca^{2+} channel blocker, on BPA-evoked release of dopamine. After PC12 cells had been incubated with 1 μM ω-CgTX or 20 μM Diltiazem for 40 min, they were incubated with 50 μM BPA for 10 min. (b) Effect of pre-treatment with ω-CgTX or with ω-AgTX, a P/Q-type Ca^{2+} channel blocker, on BPA-evoked release of dopamine. After PC12 cells had been incubated with 1 μM ω-CgTX or 1 μM ω-AgTX for 40 min, they were incubated with 50 μM BPA for 10 min Each intracellular dopamine content was measured by HPLC-ECD as described in Experimental procedures and expressed as a percentage of the control containing only vehicle. Values are means ± SEM from three different experiments. #$p < 0.05$; §$p < 0.05$; ¶$p < 0.05$; &$p < 0.05$ compared with the corresponding controls by ANOVA and post-hoc Dunnett's.

norepinephrine and 3-methoxytyramine (Greene and Rein 1978; Tuler *et al.* 1989). Neither of these metabolites summed up in both PC12 cells and media was significantly increased after treatment with 50 μM BPA for 10 min. The intracellular norepinephrine concentration after treatment with 50 μM BPA is 5.57 ± 0.08 ng/mg protein, while the control is 8.05 ± 0.21 ng/mg protein. On the other hand, the intracellular 3-methoxytyramine concentration after treatment with 50 μM BPA is 3.28 ± 0.08 ng/mg protein (control: 3.32 ± 0.03 ng/mg protein). Values are means ± SEM from three different experiments. Neither norepinephrine nor 3-methoxytyramine was distinctly detected in the media. These results indicate that the BPA-evoked loss of dopamine in PC12 cells is principally due to the release of dopamine into the medium from the cells, and not to metabolism.

Participation of N-type Ca^{2+} channels in BPA-evoked dopamine release

It is well known that the activation of voltage-gated Ca^{2+} channels tightly regulates the release of neurotransmitters from neurons. PC12 cells possess L-, N-, and P/Q-type voltage-gated Ca^{2+} channels (Liu *et al.* 1996). To determine the relative contributions if any, of these subtypes of the Ca^{2+} channel to BPA-evoked dopamine release, 20 μM diltiazem, 1 μM ω-CgTX, or 1 μM ω-AgTX as a selective L-, N- or P/Q type Ca^{2+} channel blocker, respectively, was added for 40 min prior to the addition of 50 μM BPA. A single application of each Ca^{2+} channel blocker such as 20 μM diltiazem (Fig. 3a), 1 μM ω-CgTX (Figs 3a and b) or 1 μM ω-AgTX (Fig. 3b) did not change the content of intracellular dopamine in PC12 cells, respectively, suggesting that these Ca^{2+} channel blockers do not influence the intracellular dopamine concentration by themselves. (The intracellular dopamine concentration by a single application of 20 μM diltiazem or 1 μM ω-CgTX was 381.7 ± 7.1 or 372.9 ± 22.0 ng/mg protein, respectively, and the control value was 389.4 ± 3.3 ng/mg protein in Fig. 3a. The intracellular dopamine concentration by a single application of 1 μM ω-AgTX or 1 μM ω-CgTX was 386.9 ± 9.6 or 399.5 ± 24.4 ng/mg protein, respectively, and the control value was 375.4 ± 6.9 ng/mg protein in Fig. 3b.) Pre-treatment with 1 μM ω-CgTX abolished the BPA-evoked decrease in intracellular dopamine in PC12 cells (Figs 3a and b), although, pre-treatment with 20 μM diltiazem failed to elicit such a response (Fig. 3a). In addition, the application of 1 μM ω-AgTX did not inhibit the BPA-elicited decrease in intracellular dopamine (Fig. 3b). The concentrations of 20 μM diltiazem, 1 μM ω-CgTX and 1 μM ω-AgTX are thought to be sufficient to block the opening of the L-, N-, and P/Q type Ca^{2+} channels, respectively (Taylor and Peers 1999; von Lewinski *et al.* 2003). These results collectively

suggest that the dominant route for BPA-evoked dopamine release in PC12 cells is via N-type Ca^{2+} channels.

Participation of ryanodine-sensitive calcium stores in BPA-evoked dopamine release

Calcium-induced calcium release (CICR) from ryanodine-sensitive calcium stores plays an important role in neurotransmitter release by amplifying the calcium entering through N-type Ca^{2+} channels (Smith and Cunnane 1996; Akita and Kubo 2000). It has been already known that PC12 cells have ryanodine receptors (Bennett *et al.* 1998; Koizumi *et al.* 1999; Wong *et al.* 2001). We next examined whether BPA-elicited dopamine release requires activation of ryanodine-sensitive calcium stores. A single application of neither 2 μM ruthenium red (an inhibitor of ryanodine receptors; Fig. 4a) nor 10 μM ryanodine (an inhibitor of ryanodine receptors at concentrations of more than 10 μM; Fig. 4b) altered the intracellular dopamine content of PC12 cells, suggesting that each inhibitor had no influence on the intracellular dopamine content by itself. (In Fig. 4a, the intracellular dopamine concentration of control or by a single application of 2 μM ruthenium red was 303.5 ± 16.4 or 301.3 ± 15.4 ng/mg protein and in Fig. 4b, the intracellular dopamine concentration of control or by a single application of 10 μM ryanodine was 379.7 ± 10.1 or 368.3 ± 15.6 ng/mg protein.) The BPA-evoked intracellular dopamine decrease was inhibited by treatment with ruthenium red dose-dependently (0.5–2 μM; Fig. 4a) or 10 μM ryanodine (Fig. 4b) for 40 min prior to 50 μM BPA. Ruthenium red at this concentration was reported to fully block ryanodine receptors (Xu *et al.* 1999). These observations suggest that the BPA-evoked dopamine release involved ryanodine-sensitive calcium stores in PC12 cells used in this study.

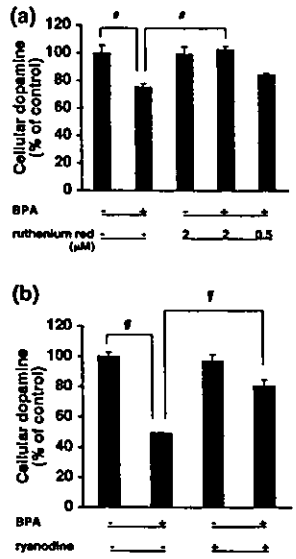

Fig. 4 Effect of ryanodine receptors on BPA-evoked release of dopamine. (a) Effect of ruthenium red, a ryanodine receptor inhibitor, on BPA-evoked release of dopamine. After PC12 cells had been incubated with 0.5 or 2 μM ruthenium red for 40 min, they were incubated with 50 μM BPA for 10 min. (b) Effect of ryanodine, a ryanodine receptor inhibitor, on BPA-evoked release of dopamine. After PC12 cells had been incubated with 10 μM ryanodine for 40 min, they were incubated with 50 μM BPA for 10 min. Each intracellular dopamine content was measured by HPLC-ECD as described in Experimental procedures and expressed as a percentage of the control containing only vehicle. Values are means ± SEM from three different experiments. ¶$p < 0.05$; #$p < 0.05$, compared with cells exposed to BPA by ANOVA and post-hoc Dunnett's.

Expression of ryanodine receptor mRNA in PC12 cells

It is widely accepted that there are three isoforms of the ryanodine receptor. To investigate the existence of ryanodine receptor mRNA in PC12 cells used in this study, RT–PCR was performed using two specific PCR primer sets for each receptor type 1–3 (Table 1). As shown in Fig. 5, the DNA fragments of appropriate size were amplified with primers for ryanodine receptor type 1 and 2, respectively. These results indicate the existence of type 1 and type 2 isoforms of the ryanodine receptor mRNA in PC12 cells used in this study.

Role of G protein and cyclic AMP pathway in BPA-evoked dopamine release

Previous studies (Baizer and Weiner 1985; Joseph *et al.* 1995) demonstrated that a cyclic AMP response was required for the release of cellular dopamine in PC12 cells. We investigated the participation of cyclic AMP in the BPA-evoked

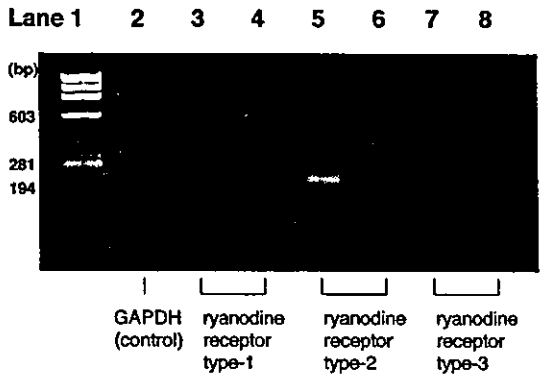

Fig. 5 The mRNA expression of ryanodine receptors in PC12 cells. The mRNA expression of each isoform of ryanodine receptors and GAPDH (control) in PC12 cells was examined by RT–PCR. Oligonucleotide primers used for PCR are listed in Table 1. Amplified cDNA products were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining under UV light. Lane 1 shows the molecular weight marker, lane 2; GAPDH, lane 3 and 4; ryanodine receptor type-1, lane 5 and 6; ryanodine receptor type-2, lane 5 and 6; ryanodine receptor type-3.

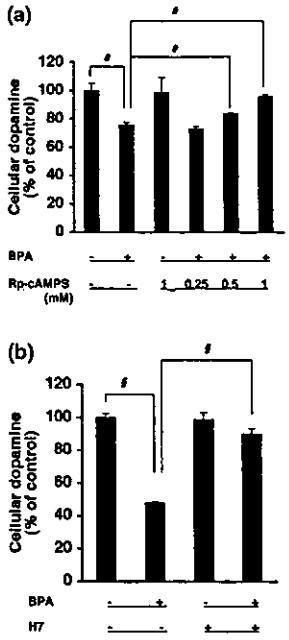

Fig. 6 Effect of pretreatment with cyclic AMP/PKA antagonist on BPA-evoked release of dopamine. (a) Effect of pre-treatment with Rp-cAMPS, a cyclic AMP antagonist, on BPA-evoked release of dopamine. After PC12 cells had been incubated with various concentration of Rp-cAMPS for 30 min, they were incubated with 50 μM BPA for 10 min. (b) Effect of pre-treatment with H7, a PKA and PKC blocker, on BPA-evoked release of dopamine. After PC12 cells had been incubated with 10 μM H7 for 30 min, they were incubated with 50 μM BPA for 10 min Each intracellular dopamine content was measured by HPLC-ECD as described in Experimental procedures and expressed as a percentage of the control containing only vehicle. Values are means ± SEM from three different experiments. #$p < 0.05$; §$p < 0.05$ compared with cells exposed to BPA by ANOVA and post-hoc Dunnett's.

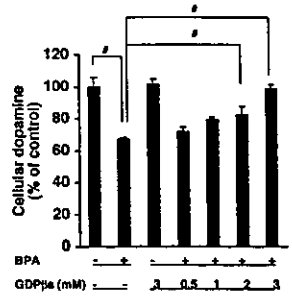

Fig. 7 Effect of pre-treatment with GDPβs, a G protein antagonist, on BPA-evoked release of dopamine. After PC12 cells had been incubated with various concentrations of GDPβs for 40 min, they were incubated with 50 μM BPA for 10 min Each intracellular dopamine content was measured by HPLC-ECD as described in Experimental procedures and expressed as a percentage of the control containing only vehicle. Values are means ± SEM from three different experiments. #$p < 0.05$, compared with cells exposed to BPA by ANOVA and post-hoc Dunnett's.

dopamine release. Although a single application of 1 mM Rp-cAMPS, cyclic AMP antagonist, did not change the intracellular dopamine content (In Fig. 6a, the intracellular dopamine concentration of control or by a single application of 1 mM Rp-cAMPS was 303.5 ± 16.4 or 311.3 ± 18.5 ng/mg protein.) Pre-treatment with Rp-cAMPS dose-dependently (0.25–1 mM) inhibited the BPA-evoked reduction of the intracellular dopamine content (Fig. 6a). Similarly, treatment with 10 μM H7, protein kinase A (PKA) and protein kinase C (PKC) inhibitor (Fig. 6b) or 100 μM H89, PKA inhibitor (data not shown) for 30 min prior to the addition of 50 μM BPA inhibited the BPA-evoked decrease in the intracellular dopamine in PC12 cells. In Fig. 6(b), the intracellular dopamine concentration of control or by a single application of 10 μM H7 was 379.7 ± 10.1 or 378.3 ± 26.4 ng/mg protein. Rp-cAMPS, H7 and H89 at the concentrations used in this study were reported to be able to inhibit cyclic AMP/PKA (Hirsh *et al.* 1990; Snyder *et al.* 1992; Perets *et al.* 1996). Subsequently, in order to prove whether the BPA-evoked dopamine release requires activation of membrane receptors, GDPβs, an antagonist of GTP at the G protein-binding site, was used for pre-treatment for 30 min prior to the application of 50 μM BPA. GDPβs dose-dependently (0.5–3 mM) inhibited the BPA-evoked dopamine release (Fig. 7). A single application of 3 mM GDPβs did not alter the intracellular dopamine content. In Fig. 7, the intracellular dopamine concentration of control or by a single application of 3 mM GDPβs was 372.2 ± 22.1 or 378.5 ± 11.2 ng/mg protein, suggesting that GDPβs did not influence the intracellular dopamine concentration by itself. The concentrations of GDPβs are adequate to inhibit G protein (Brass *et al.* 1986). These results demonstrate that G protein and the cyclic AMP pathway are required for the BPA-evoked release of dopamine.

Discussion

Using a variety of approaches, we have provided evidence that BPA regulates intracellular dopamine content. The mode of action of BPA, according to the findings reported herein, appears to be at the level of secretion. Neither clorgyline nor pargyline, MAO inhibitor, were capable of inhibiting the BPA-evoked diminution of intracellular dopamine content (Fig. 2) and BPA did not increase the levels of minor metabolites of dopamine such as norepinephrine and 3-methoxytyramine, thus ruling out the possibility that the decrease in the intracellular dopamine was due to cellular metabolism.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine biosynthesis. Intracellular dopamine increases with an elevation of the mRNA of TH for hours and by the phosphorylation of TH via cyclic AMP-dependent PKA

for several minutes (Haycock 1996; Salvatore *et al.* 2001). To exclude the genomic influence of the mRNA, short-term exposure (10 min) to BPA was adopted. Cyclic AMP antagonists can interfere with the phosphorylation of TH and decrease the synthesis of intracellular dopamine, however, as shown in Fig. 6(a), Rp-cAMPS, a cyclic AMP antagonist, inhibited the BPA-evoked intracellular dopamine decrease, suggesting that the short-term phosphorylation of TH did not significantly influence the alteration of intracellular dopamine content in this experiment. For these reasons, we concluded that the BPA-elicited intracellular dopamine decrease in PC12 cells was mainly dependent upon the release of dopamine.

In this study, PC12 cells were treated with 25–150 μM BPA. This dose of BPA is thought to be comparable with those to which humans might be exposed in daily life, for example, BPA concentration in saliva after dental treatment with sealants (Olea *et al.* 1996).

Acidity evokes catecholamine secretion via activation of N- and P/Q- type voltage-gated Ca^{2+} channels in PC12 cells (Taylor *et al.* 1999). To avoid the influence of acidity, we used media with a pH of 7.4. On the other hand, except under acidic conditions, the dopamine in the medium can decompose, in other words, there is a possibility of inaccurate measurements of dopamine concentrations at pH 7.4. In order to evaluate the delicate change of dopamine release when chemicals modulating signal transductions were added prior to BPA, we measured intracellular dopamine content instead of the dopamine concentration in the medium due to secretion.

Pre-treatment with ω-CgTX, selective N-type Ca^{2+} channel blocker, abolished the BPA-evoked release of dopamine (Figs 3a and b). From this result, the dominant route for BPA-evoked release of dopamine appears to be via N-type Ca^{2+} channels. These findings are in accordance with previous results as follows; PC12 cells possess N-, L-, and P/Q-type voltage-gated Ca^{2+} channels (Liu *et al.* 1996). Although each subtype significantly contributed to Ca^{2+} influx following deporalization, in the case of dopamine exocytosis, P/Q-type channels were not involved and L-type channels played only a minor role. The N-type was primarily responsible for triggering the exocytosis of dopamine in PC12 cells (Taylor *et al.* 1999).

The BPA-evoked release of dopamine was inhibited by pretreatment with ryanodine receptor blockers such as ruthenium red and ryanodine (Figs 4a and b). Thus the data in Figs 4(a and b) suggest that the BPA-evoked release of dopamine involves the activation of ryanodine-sensitive calcium stores in PC12 cells. We also confirmed the existence of type 1 and type 2 ryanodine receptor mRNA in PC12 cells (Fig. 5). The type 1 ryanodine receptor is well known to be important in sustaining excitation-contraction coupling in skeletal fibers. The type 2 ryanodine receptors activate neurosecretory pathways in PC12 cells (Clementi *et al.* 1996). In the present study, the BPA-evoked dopamine release may have been activated by the type 2 ryanodine receptors in PC12 cells.

Pre-treatment with Rp-cAMPS, a cyclic AMP antagonist (Fig. 6a), H7, a PKA and PKC inhibitor (Fig. 6b) or H89, a PKA inhibitor (data not shown) for 30 min prior to 50 μM BPA inhibited the BPA-evoked dopamine release, indicating that the cyclic AMP/PKA pathway is required for the BPA-evoked dopamine release in PC12 cells. Cyclic AMP/PKA activation contributes to the stimulation of the ryanodine receptor, which produces Ca^{2+} spikes, by cyclic AMP-dependent phosphorylation in pancreatic β cells (Islam *et al.* 1998). Also in the nervous system, ryanodine receptors are regulated by cyclic AMP-dependent phosphorylation (Yoshida *et al.* 1992). On the other hand, cyclic AMP-dependent PKA phosphorylates L-type Ca^{2+} channels in cardiac muscle cells (Yatani *et al.* 1989) and in pancreatic β cells (Holz *et al.* 1999). In the peripheral neurons, cyclic AMP-stimulated Ca^{2+} efflux and acetylcholine release appears to be mediated mainly by N-type Ca^{2+} channels (Kojima *et al.* 1997). In PC12 cells, it has been known that cyclic AMP-dependent PKA linked to N-type Ca^{2+} channels (Solem *et al.* 1997) and the activation of cyclic AMP/PKA pathway stimulates dopamine release (Baizer and Weiner 1985; Joseph *et al.* 1995). As BPA activates cyclic AMP and cyclic AMP-dependent PKA in PC12 cells, this may possibly phosphorylate ryanodine receptors and N-type Ca^{2+} channels.

BPA-evoked dopamine release from PC12 cells was blocked by GDPβs, a G protein inhibitor (Fig. 7), suggesting that G proteins are likely involved in the BPA-evoked dopamine release. Collectively, our data support a potential model for the action of BPA in PC12 cells as illustrated in Fig. 8. Our investigation showed that the BPA-evoked dopamine release required G protein and cyclic AMP-dependent PKA. PKA may stimulate N-type Ca^{2+} channels by phosphorylation (Kojima *et al.* 1997; Solem *et al.* 1997). CICR may be elicited both by Ca^{2+} influx through N-type Ca^{2+} channels (Smith and Cunnane 1996; Akita and Kubo 2000) and by phosphorylations of ryanodine receptors via PKA (Yoshida *et al.* 1992; Islam *et al.* 1998). But the model presented in Fig. 8 does not take into account the role of inositol 1,4,5-triphosphate receptors as mediators of signal transduction. On the other hand, BPA stimulates nitric oxide synthesis in mouse endothelial cells (Noguchi *et al.* 2002) and the type 2 ryanodine receptor is activated by a nitric oxide/cyclic GMP pathway in PC12 cells (Clementi *et al.* 1996). It is possible that the BPA-evoked release of dopamine is partially dependent upon the nitric oxide/cyclic GMP pathway.

The mechanisms of action of steroid hormones can be classified as genomic or non-genomic. The long-term genomic effects of estrogen are due to the binding of estrogen to intracellular receptors. The activated estrogen receptor complex then acts as a transcription factor that

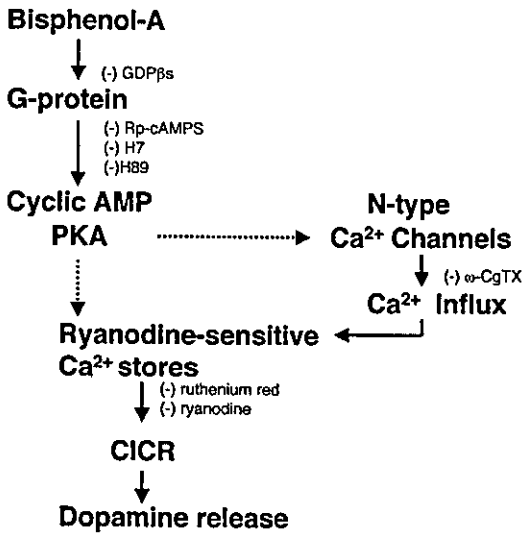

Fig. 8 A proposed model for the non-genomic action of BPA in PC12 cells. In this experiment, we demonstrated the participation of G-protein, cyclic AMP/PKA, N-type Ca^{2+} channels, and ryanodine-sensitive Ca^{2+} stores in BPA-evoked dopamine release. Cyclic AMP-dependent PKA may be linked to N-type Ca^{2+} channels (Kojima *et al.* 1997; Solem *et al.* 1997) and phosphorylate ryanodine receptors (Yoshida *et al.* 1992; Islam *et al.* 1998).

induces the transcription of target genes and protein synthesis. Regarding non-genomic actions, 17β-estradiol inhibits norepinephrine secretion which is independent of signaling pathways involving phospholipase-C (PLC), PKA or PKC, but dependent on inhibition of L- and N-type Ca^{2+} channels and $P2X_2$ receptors (Kim *et al.* 2000). Many studies have reported that BPA has structural homology with a ring of β-estradiol and is suspected to bind to nuclear estrogen receptors thus mimicking the genomic actions of estrogens. In this study, we demonstrated that BPA induced dopamine release via a cyclic AMP/PKA pathway and N-type Ca^{2+} channels, suggesting that BPA acts through different pathways from 17β-estradiol in a non-genomic manner. Recently, it was demonstrated that BPA disrupted thyroid hormone action (Moriyama *et al.* 2002). Thus BPA probably acts on various types of receptors.

Other endocrine disruptor PCBs are cholinergic muscarinic receptor agonists known to stimulate PLC to produce second messengers such as inositol 1,4,5-triphosphate, which releases intracellular Ca^{2+} from the endoplasmic reticulum, and diacylglycerol, which activates PKC (Kodavanti *et al.* 1994). In addition, Ortho-substituted PCBs rapidly mobilize Ca^{2+} by an interaction with ryanodine receptors in heart, skeletal muscle (Wong and Pessah 1996) and PC12 cells (Wong *et al.* 2001). However, the exposure to PCBs for less than an hour inhibits the spontaneous release of dopamine and significantly increases the intracellular dopamine concentration in PC12 cells (Seegal *et al.* 1989). Fenthion, an organophosphate pesticide, does not increase dopamine release either (Tuler *et al.* 1989). From these findings including our results, BPA appears to act via different pathways from other ubiquitious environmental contaminants such as PCBs or Fenthion in a non-genomic manner.

In conclusion, we showed that BPA stimulates dopamine release in a non-genomic manner in PC12 cells. *In vivo*, chronic pre-natal and neonatal exposure to BPA leads to the upregulation of central dopaminergic neurotransmission by increasing dopamine D1 receptors in a genomic manner (Suzuki *et al.* 2003). Though we do not think we can simply compare and/or combine these data, we think that BPA has an influence on dopaminergic functions.

Acknowledgements

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Neurosteroid-induced hyperalgesia through a histamine release is inhibited by progesterone and *p,p'*-DDE, an endocrine disrupting chemical

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Abstract

The intraplantar injection of dehydroepiandrosterone sulfate (DHEAS), a representative neurosteroid, showed hyperalgesia in the Hargreaves' thermal or automatic paw-pressure mechanical nociception test. The DHEAS-induced hyperalgesia was abolished by diphenhydramine (DPH), a H_1 histamine (*His*) receptor antagonist, as well as the hyperalgesia induced by *His* or compound 48/80, a mast cell degranulating agent. The DHEAS-induced hyperalgesia was also blocked by progesterone (PROG), another type of neurosteroid and a putative neurosteroid receptor antagonist. Neither DPH nor PROG showed any changes in the thermal threshold. On the other hand, endocrine disrupting chemicals (EDCs) are known to disrupt reproductive system in wild-lives and humans through the disturbance of the endocrine homeostasis. In this study, the flexor responses induced by intraplantar injection of DHEAS were blocked by *p,p'*-DDE, an EDC as well as by PROG in the algogenics-induced nociceptive flexor responses test (ANF test) in mice. Similarly, *p,p'*-DDE blocked the DHEAS-induced hyperalgesia in Hargreaves' thermal nociception test. Besides the hyperalgesic actions, DHEAS increased vascular permeability as measured with Evans blue plasma extravasation. Consistent with behavioral studies, it was blocked by DPH, PROG, and *p,p'*-DDE. These results suggest that DHEAS has significant hyperalgesic and vasodilatory actions through histamine release, and these actions were reversible by PROG and an EDC.

Keywords: Neurosteroid; Endocrine disrupting chemical; Pain; Hyperalgesia; Vasodilatation

1. Introduction

The steroids synthesized de novo in the nervous system are called neurosteroids, and have a wide variety of diverse functions (Baulieu, 1998; Rupprecht and Holsboer, 1999). It has been known that neurosteroids exert their actions not through classical steroid hormone intracellular receptors, but also through plasma membrane receptors (Compagnone and Mellon, 2000; Mellon and Griffin, 2002). The latter mechanisms include the allosteric actions on ligand-gated channels such as $GABA_A$ (Majewska, 1999), NMDA (Gibbs et al., 1999) and nicotinic receptors (Buisson and Bertrand, 1999). In addition to these findings, however, a series of reports has shown that σ-receptor, a drug receptor, might be another target of the neurosteroids (Maurice et al., 1999; Ueda et al., 2001a).

Most recently, we reported that pregnenolone sulfate and dehydroepiandrosterone sulfate, two representative neurosteroids, induced flexor responses when given intraplantarly in mice (Ueda et al., 2001b). The underlying mechanisms seem to be mediated through two distinct sites of action. One is through a direct stimulation of sensory neurons, and higher doses of neurosteroids are required for this mechanism. The other one is likely through a histamine (*His*) release from mast cells, and very low doses of neurosteroids are enough to show the responses. So the latter mechanism seems to be better targets for sensory stimulation and its inhibition. In the present study, we examined the modulatory effects of neurosteroids on several types of nociception. We also report that neurosteroid increases vascular permeability through histamine release. On the other hand, endocrine disrupting chemicals (EDCs) are known to mimic the actions of various steroid hormones, and cause serious dysfunction of reproductive organs (McLachlan, 2001). Here, we also report the antagonistic activity of *p,p'*-DDE, an EDC, on the neurosteroid-induced hyperalgesia and plasma extravasation.

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2. Materials and methods

2.1. Animals

Male ddY mice weighing 20–22 g were used in all experiments. Procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983).

2.2. Algogenics-induced biting and licking responses test (ABL test)

One hour prior to the intraplantar (i.pl.) injection of test drugs, animals were adapted to an individual plastic cage, which served as the observation chamber. Immediately following the i.pl. injection, each mouse was placed into the transparent cage over a mirror and behavioral testing was begun. Biting and licking behaviors for 20 min after i.pl. injection were evaluated as nocifensive responses. The total amount of time to show the nocifensive responses was measured in 5-min intervals for 20 min.

2.3. Hargreaves' test

Hargreaves' test was performed according to Hargreaves et al. (1988). A thermal beam was focused on the hind limb footpads of mice placed on a glass surface and the withdrawal–response latency was measured with a 20-s cut-off time.

2.4. Paw-pressure test

Paw-pressure test was performed as described previously (Dobolyi et al., 2002). Mice were placed into a Plexiglas chamber on a wire mesh grid floor and were allowed to accommodate for a period of 1 h. The mechanical stimulus was then delivered onto the middle of the plantar surface of the right hind paw using a 0.8–0.9-mm diameter filament connected to an automatic Transducer Indicator (Model 1601, IITC Inc., Woodland Hills, USA). The filament used produces 10 g of force at 5 s, when paw withdrawal is elicited in naive mice. In this experiment, a cut-off pressure of 20 g was set to avoid tissue damage.

2.5. ANF test

Experiments were performed as described previously (Inoue et al., 1998; Ueda et al., 2001b; Dobolyi et al., 2002). Mice were held in a suspended cloth sling. Test agents were delivered through two polyethylene cannulae inserted into the plantar surface of the right hind limb, which was connected to an isotonic transducer/recorder. For normalization, the largest spontaneous response occurring immediately after cannulation was considered the maximal withdrawal force for each animal. Nociceptive activity was expressed as the ratio of the test drug-elicited force to the maximal force in each mouse. In the dose–response experiments, increasing doses were given at 5-min intervals, with each dose administrated twice. Neurosteroid was given i.pl. 10 and 5 min prior to and 5, 10, 20, and 30 min after vehicle or antagonist injection. All animals were used for only one experiment by the observer who did not know what kind of pre-treatments had been given.

2.6. Evans blue plasma extravasation

Experiments were performed as reported previously (Singh et al., 1999; Naveilhan et al., 2001). Briefly, mice were anaesthetized with pentobarbital (50 mg kg^{-1}, i.p.) and injected intravenously with Evans blue (50 mg kg^{-1}) into the tail vein 10 min before i.pl. injection of test drugs in 10 μl. The other paw was injected with vehicle. The pre-treatment drugs were injected first and allowed to remain in the skin for 5 min. After 30 min, the plantar skin of the paw was removed, dried of excess liquid, weighed and incubated in formamide for 24 h at 55 °C. Extravasated Evans blue was measured by spectrophotometer at 620 nm. Results are expressed as the ratio of arbitrary fluorescence units for Evans blue of drug-treated paw to that of vehicle-treated paw.

2.7. Drugs

Histamine, compound 48/80, DHEAS, and PROG were obtained from Sigma (St. Louis, MO). Diphenhydramine hydrochloride (DPH) was obtained from Nacalai Tesque (Kyoto, Japan). *p,p'*-DDE was obtained from KANTO KAGAKU (Tokyo, Japan). Evans blue was obtained from WAKO (Osaka, Japan). All drugs were dissolved in physiological saline, and were administrated by i.pl. injection in a volume 20 μl in cases with ABL-, Hargreaves'-, and paw-pressure tests, while 2 μl in case with ANF test.

2.8. Statistical analysis

Statistical evaluations were performed using the Student's *t*-test following comparison with repeated measures ANOVA. Significance was established at $P < 0.05$. All results are expressed as the mean ± S.E.M.

3. Results

The intraplantar (i.pl.) injection of histamine (*His*) and compound 48/80 at a dose of 100 pmol and 1 μg, respectively showed marked nocifensive responses, such as biting and licking. Fig. 1 shows the total time showing such nocifensive behaviors during each 5 min throughout 20 min. The nocifensive responses for each case were maximal at the period between 0 and 5 min after the injection, and fell down as early as the period between 5 and 10 min. Among the data with *His* (1 pmol to 1 nmol), the maximum response was

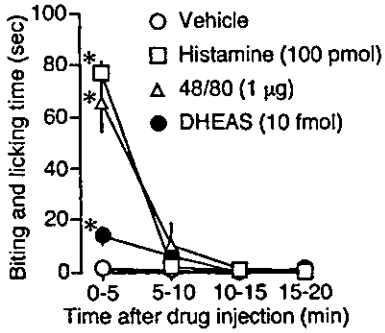

Fig. 1. Time course of DHEAS-induced biting and licking behaviors. The total time to show biting and licking behaviors, which was measured in 5-min intervals for 20 min after i.pl. injection of vehicle, *His* (100 pmol), compound 48/80 (1 μg) or DHEAS (10 fmol) are shown. Data represent the mean ± S.E.M. from six or more separate experiments (*$P < 0.05$).

observed with the 100 pmol (data not shown). The nocifensive action by DHEAS (i.pl.) at 10 fmol resembled these two cases, but the potency was much weaker.

In the Hargreaves' thermal nociception test, we measured the latency (in seconds) to show withdrawal responses from the heat stimulation given to the paw of hind limb. Average of threshold was 10.47 ± 0.11 s in vehicle-treated mice. The intraplantar injection of *His* (100 pmol) to the ipsilateral side of limb decreased the threshold as early as 10 min after the injection, and lasted for almost 60 min (Fig. 2A). Compound 48/80 (1 μg, i.pl.) and DHEAS (10 fmol, i.pl.) also showed very similar hyperalgesic actions (Fig. 2B and C). In the paw-pressure test, nociceptive threshold was represented as the threshold (in grams) to show withdrawal responses against the mechanical stimulation. As shown in Fig. 2D–F, however, all these three compounds at the corresponding doses showed significant, but weaker nociception

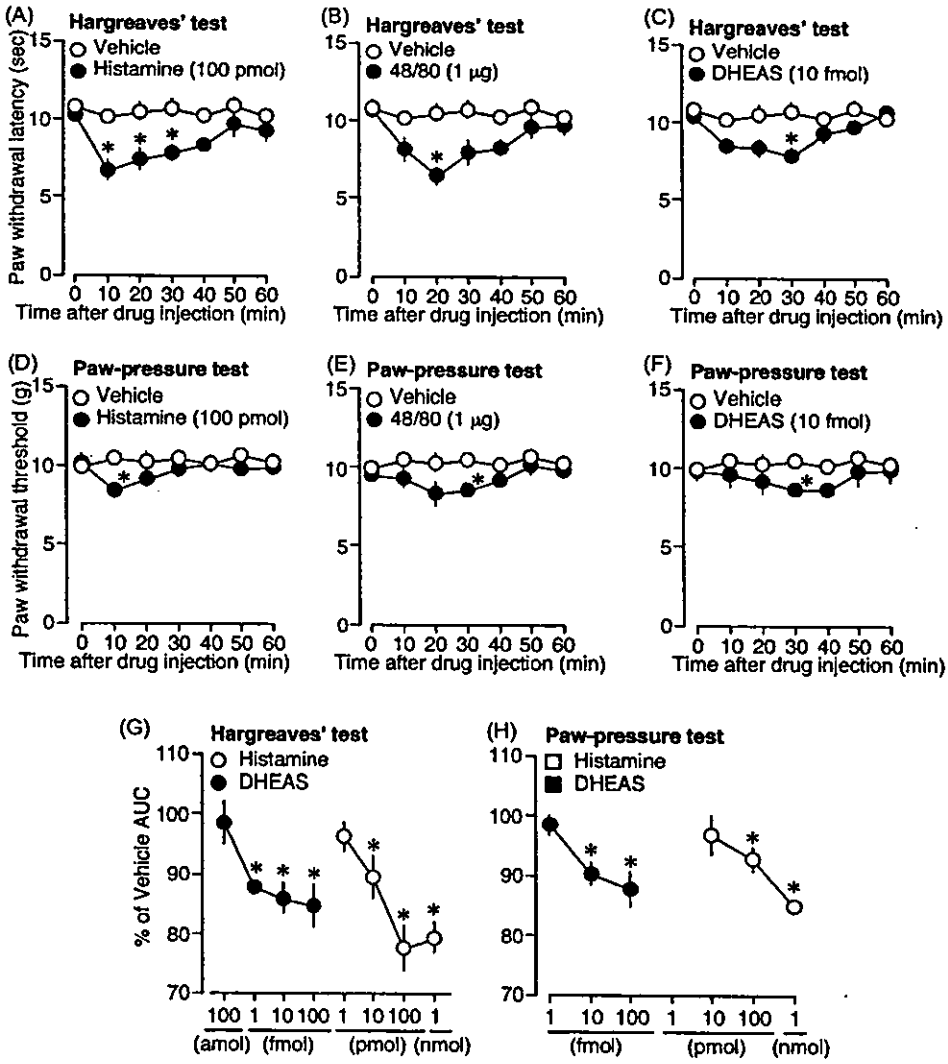

Fig. 2. DHEAS-induced hyperalgesia to thermal and mechanical stimuli. Hargreaves' (radiant heat directed to a paw) and paw-pressure tests were performed as described in Section 2. Time courses of withdrawal responses after injection of vehicle, *His* (100 pmol), compound 48/80 (1 μg) or DHEAS (10 fmol) are shown (A–F). In both tests, areas under the curve (AUC) over 60 min after drug injection are shown (G and H). Data represent the mean ± S.E.M. from six or more separate experiments (*$P < 0.05$).

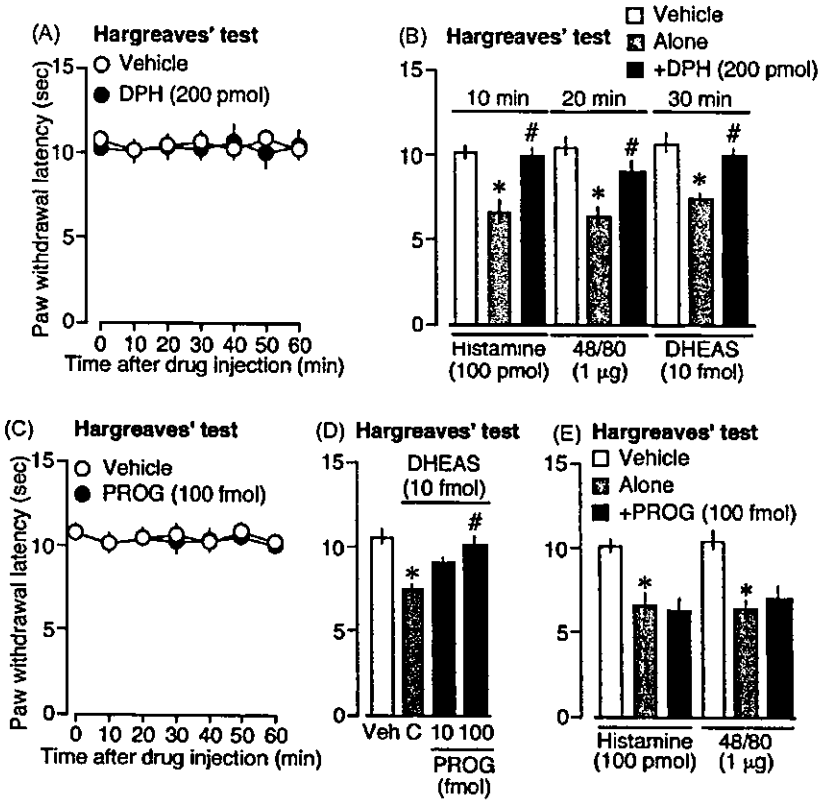

Fig. 3. Inhibitory action of progesterone on DHEAS-induced thermal hyperalgesia through a histamine release. Time course of DPH (200 pmol, i.pl.) is shown with Hargreaves' test (A). Responses after intraplantar application of vehicle, *His* (100 pmol), compound 48/80 (1 μg) and DHEAS (10 fmol) are shown at each time (10 min, 20 min and 30 min, respectively) when maximal response was observed. $^{*}P < 0.05$ compared with vehicle-treated group. $^{\#}P < 0.05$ compared with *His*-, 48/80- or DHEAS-treated groups. (B). Time course of PROG (100 fmol, i.pl.) and its inhibition of DHEAS-induced hyperalgesia are shown. $^{*}P < 0.05$ compared with vehicle-treated group. $^{\#}P < 0.05$ compared with DHEAS-treated group. (C and D). No effects on *His*- and compound 48/80-induced hyperalgesia by PROG. $^{*}P < 0.05$ compared with vehicle-treated group. (E). Data represent the mean ± S.E.M. from six or more separate experiments ($^{*}P < 0.05$).

than those in the thermal test. Fig. 2G and H demonstrated the comparisons of hyperalgesia by *His* and DHEAS by use of area under the curve (AUC) analysis. In both tests, *His* and DHEAS-induced hyperalgesia in a dose-dependent manner.

Diphenhydramine (DPH), a known H_1 antagonist of *His* receptor had no effects on the thermal nociception, as shown in Fig. 3A. Coadministration of DPH (200 pmol, i.pl.) completely abolished all the hyperalgesia induced by these three compounds (Fig. 3B). On the other hand, 100 fmol (i.pl.) of progesterone (PROG), a putative neurosteroid antagonist candidate (Ueda et al., 2001a,b) also had no effects on this test by itself (Fig. 3C), but completely abolished the DHEAS-induced hyperalgesia (Fig. 3D). However, PROG (100 fmol, i.pl.) could not block *His*- and compound 48/80-induced hyperalgesia (Fig. 3E), confirming that PROG would be a specific antagonist of DHEAS.

As previously reported (Ueda et al., 2001b), DHEAS showed "nociceptive" responses in the algogenics-induced nociceptive flexor responses test (ANF test) in mice at a very small dose. The intraplantar injection of DHEAS at a dose of 1 fmol caused transient flexor responses, and the repeated injections of this compound showed constant responses for 30 min. It has been reported that *p,p′*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene), a metabolite of DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane), is a candidate of endocrine disrupting chemicals (Kelce et al., 1995; McLachlan, 2001). When this compound at a dose of 10 fmol was co-injected with DHEAS (1 fmol, i.pl.), the flexor responses were abolished (Fig. 4B). In the thermal nociception test, on the other hand, the hyperalgesic action by DHEAS was also inhibited by *p,p′*-DDE (Fig. 4D). However, *p,p′*-DDE, when injected alone, it did not produce significant responses in both cases (Fig. 4A and C).

His has been known as a vasoactive molecule. Thus, we next examined whether DHEAS could increase vascular permeability by extraction of extravasated Evans blue. As shown in Fig. 5A and B, DHEAS (10 fmol, i.pl.) markedly increased vascular permeability. Administration of DPH (200 pmol, i.pl.) before i.pl. injection of DHEAS completely inhibited plasma extravasation (Fig. 5C), indicating that DHEAS induces vasodilatation through histamine release. Moreover, DHEAS-induced plasma extravasation was largely reduced by pre-treatment with PROG (100 fmol,

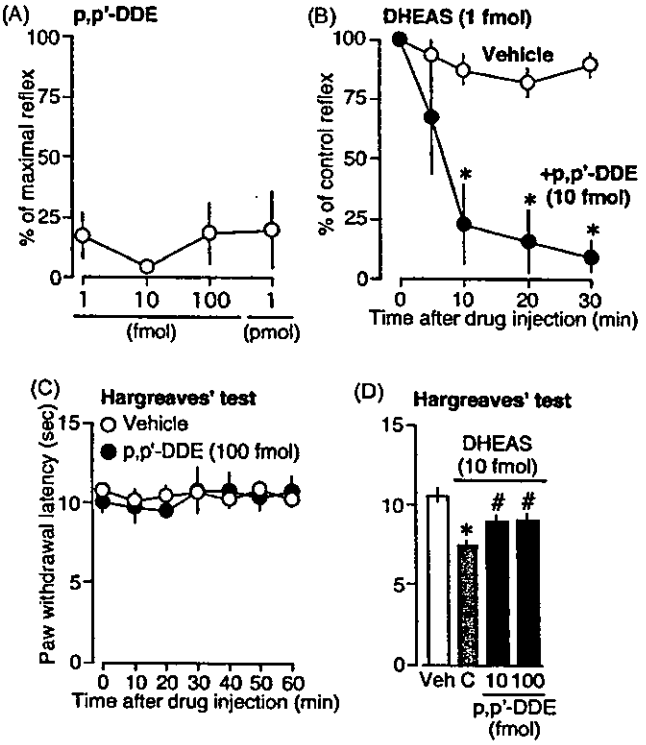

Fig. 4. Antagonistic activity of *p,p'*-DDE on DHEAS-induced nociceptive responses. Dose–response curve of *p,p'*-DDE in the ANF test (A). Responses by intraplantar application of DHEAS (1 fmol) and DHEAS (1 fmol) plus *p,p'*-DDE (10 fmol) (B). Time course of *p,p'*-DDE (100 fmol, i.pl.) is shown with Hargreaves' test (C). Responses by intraplantar application of vehicle, DHEAS (10 fmol) and DHEAS (10 fmol) plus *p,p'*-DDE (100 fmol). Results represent the latency (in seconds) at 30 min after the injection (D), as the mean ± S.E.M. from six or more separate experiments ($^{*}P < 0.05$). $^{*}P < 0.05$ compared with vehicle-treated group. $^{\#}P < 0.05$ compared with DHEAS-treated group.

i.pl.) and *p,p'*-DDE (100 fmol, i.pl.), while PROG and *p,p'*-DDE had no effect by themselves (Fig. 5B and D).

4. Discussion

In several reports including ours (Koda et al., 1996; Ueda and Inoue, 2000), *His*-induced nociception and hyperalgesia when locally applied, and the receptor involved is revealed to be H_1 subtype. As DPH (200 pmol) itself was ineffective on thermal sensation, it is evident that the *His* release is not working to determine the thermal threshold. However, intraplantarly administered *His* caused marked, but transient nocifensive responses and potentiation of thermal nociception. As shown in Figs. 1 and 2A, *His* showed significant thermal hyperalgesia for 30 min, even after the cessation of transient nocifensive responses for initial 5 min. Similar, but weaker *His*-induced hyperalgesia was also observed in the paw-pressure test (Fig. 2D and H). In our preliminary study, *His*-induced nociception in the ANF test was clearly blocked by intrathecal injection of CP-99994, an NK1 substance P receptor antagonist, while this antagonist did not affect the nociception in the paw-pressure test. Thus, it is very likely that the difference of nociceptive fibers involved in these two nociception tests causes this difference of *His* sensitivity. Although the maximal degree of hyperalgesia was limited, but DHEAS showed more potent thermal and mechanical hyperalgesic action than *His*, since the DHEAS dose required for equivalent actions was 10,000 times lower than the *His* (Fig. 2G and H). Altogether, with the finding that DHEAS-induced hyperalgesia was completely blocked by DPH, it is suggested that the amplification through a *His* release would be working in this mechanism.

It has been known that mast cells contain vasoactive molecules such as histamine, and mast cell degranulating agents such as compound 48/80 increase vascular permeability. Evans blue are known to bind to albumin, and used as an indicator of vascular permeability in general. In Evans blue extravasation experiments, DHEAS showed a marked increase in vascular permeability, and this was completely blocked by DPH (Fig. 5A–C). These results also provide the evidence supporting that DHEAS induces a histamine release.

Previously, we demonstrated that neurosteroids modulate [^{35}S]GTPγS binding in brain membranes, and induce nociception (Ueda et al., 2001a,b). In both experiments, DHEAS acted as an agonist, while PROG did act as an antagonist. Such relation were also observed elsewhere (Maurice et al., 2001). In the present study, PROG markedly blocked DHEAS-induced hyperalgesia, while it had no effect on *His*-/compound 48/80-induced ones (Fig. 3D and E). Similarly, DHEAS-induced plasma extravasation was largely

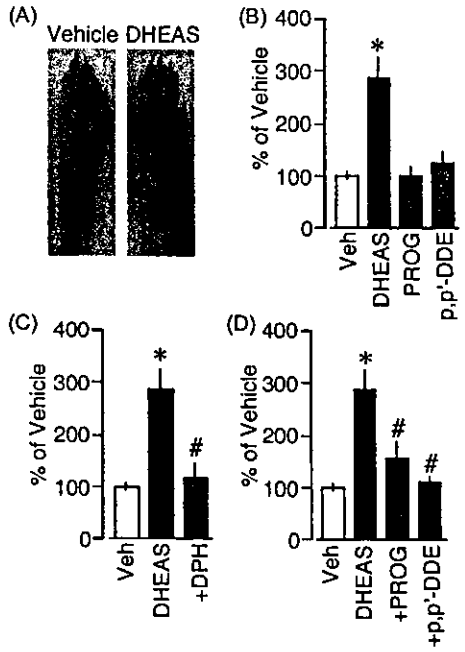

Fig. 5. DHEAS-induced plasma extravasation through histamine release. Paws of mice 30 min after i.pl. injection of DHEAS (10 fmol) or vehicle (A). Quantification of Evans blue extravasation after DHEAS (10 fmol), PROG (100 fmol), p,p'-DDE (100 fmol) or vehicle (B). DHEAS (10 fmol)-induced vascular permeability in the presence or absence of DPH (200 pmol), PROG (100 fmol) or p,p'-DDE (100 fmol) (C,D). Results are expressed as the ratio of arbitrary fluorescence units for Evans blue of drug-treated paw to that of vehicle-treated paw, and represent as the mean ± S.E.M. from six or more separate experiments ($^*P < 0.05$). $^*P < 0.05$ compared with vehicle-treated group. $^\#P < 0.05$ compared with DHEAS-treated group.

reduced by PROG (Fig. 5D). These results strengthen our classification that PROG is a specific antagonist of DHEAS.

Another issue to be discussed is the physiological significance of these neurosteroid-induced actions on nociception. The doses of neurosteroids required for such actions are low enough to work in vivo, since the local concentration of DHEAS injected (10 fmol/20 μl) is comparable with that in the plasma level (1 nM) (Robel et al., 1999). Thus, it is possible that the in vivo *His* release and following influence on the nociceptive threshold are regulated by DHEAS. However, as shown in Fig. 3C, PROG itself had no effect on the basal threshold in the Hargreaves' test, though PROG could antagonize the DHEAS actions. Thus, it is unlikely that plasma levels of DHEAS are working in vivo in the stimulation of nociceptive fibers, though the possibility remains that DHEAS released upon local injury might affect the nociceptive threshold.

The next issue in this study is about the antagonism of DHEAS actions by p,p'-DDE. It is believed that the major toxicity of endocrine disrupting chemicals is related to the disturbance of sex steroid hormone homeostasis. One of the best known examples is the pesticide, DDT. Although the toxicity of DDT for the health of humans and animals is well known (Kupfer and Bulger, 1980), p,p'-DDE, a metabolite of DDT, is also thought to be dangerous in the sense of disturbance of endocrine homeostasis. This compound is reported to possess strong steroid modulatory actions (Kelce et al., 1995; McLachlan, 2001). In the present study, we examined whether p,p'-DDE might attack the novel neurosteroid actions through a possible histamine release. p,p'-DDE blocked the DHEAS-induced flexor responses in the ANF test, at a dose of as low as 10 fmol. This compound also blocked the DHEAS-induced hyperalgesia and plasma extravasation (Figs. 4D and 5D). As mentioned above, the hyperalgesia is likely mediated through a *His* release, possibly from mast cells. Thus, the present study may be the first demonstration of p,p'-DDE action on the nervous system through indirect mechanisms.

In conclusion, we demonstrated the neurosteroid-induced regulation of nociceptive threshold and vascular permeability, and some influences of p,p'-DDE on it. We also demonstrated that the neurosteroid-induced regulation may be mediated through a *His* release from mast cells.

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Bisphenol A, an environmental endocrine-disrupting chemical, inhibits hypoxic response via degradation of hypoxia-inducible factor 1α (HIF-1α): structural requirement of bisphenol A for degradation of HIF-1α☆

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Abstract

Bisphenol A (BpA), an endocrine-disrupting chemical, is known to be a xenoestrogen and to affect the reproductive functions of animals. Recent reports have documented BpA-induced developmental abnormalities in the neuronal systems of humans and animals, and these effects appear to be non-estrogenic. In this study, we found that BpA inhibited the hypoxic response of human hepatoma cells. The expression of hypoxic response genes such as the erythropoietin (EPO) gene is done via a hypoxia inducible factor 1 (HIF-1)-dependent signaling pathway. To investigate possible structural requirements for this inhibitory effect, several BpA analogs were synthesized and added to this system. The blocking of two phenol groups in BpA did not change the effect, but the inhibition completely disappeared by the removal of two central methyl groups in BpA (the resulting compound is designated BpF). BpA, but not BpF, promoted degradation of the HIF-1α protein, which is a component of HIF-1, followed by inhibition of EPO induction. An immunoprecipitation assay indicated that BpA dissociated heat shock protein 90 (Hsp90) from HIF-1α and destabilized HIF-1α protein. HIF-1α is usually degraded first by ubiquitination and then by the proteasome pathway. Cobalt ion inhibits ubiquitination of HIF-1α and stabilizes it. In the present study, BpA promoted HIF-1α degradation in the presence of cobalt and in the presence of proteasome inhibitor. These results suggest that BpA degraded HIF-1α via a currently unknown pathway, and that this phenomenon required two methyl groups in BpA.

Keywords: Bisphenol A; HIF-α; Hsp90; Erythropoietin

Bisphenol A (BpA) is widely used in industry and dentistry and is one of the most common environmental endocrine disruptors. BpA first became a topic as a xenoestrogen, and there have been many reports on its disrupting effect on estrogenic or sex-related function [1]. Recently, non-estrogenic effects of BpA on the central nervous system have been reported. In the early development of *Xenopus laevis*, BpA has been shown to induce apoptosis in central nervous tissue cells of the brain and spinal cord [2]. In mice, prenatal and neonatal exposure to BpA induces a significant increase in the levels of dopamine D_1 receptor mRNA in the whole brain and enhances central dopamine D_1

☆ *Abbreviations:* BpA, bisphenol A; BpE, bisphenol E; BpF, bisphenol F; BpA-OMe, 2,2-bis(4-methoxyphenyl)propane (dimethyl BpA); HIF-1, hypoxia-inducible factor 1; EPO, erythropoietin; Hsp90, heat shock protein; pVHL, von Hippel–Lindau; Arnt, aryl hydrocarbon nuclear translocator; VEGF, vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate.

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receptor-mediated activity [3]. Despite these intriguing results, however, the molecular mechanism by which BpA induces these phenomena remains unclear.

Hypoxia-inducible factor 1α (HIF-1α) responds to hypoxia by binding to the hypoxia response element (HRE) of target genes, including erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glucose transporter genes [4]. HIF-1α binds HRE motifs as a heterodimeric complex with an aryl hydrocarbon nuclear translocator (Arnt). Both HIF-1α and Arnt are members of a novel subclass of the basic helix–loop–helix (bHLH) family of transcription factors. During normoxia, HIF-1α is hydoxylated on its proline residue by prolyl hydroxylases, resulting in its ubiquitination by von Hippel–Lindau tumor suppressor protein (pVHL) and degradation by proteasome [5,6]. During hypoxia, proline hydroxylation is inhibited and HIF-1α is not ubiquitinated. As a result, HIF-1α protein accumulates and regulates HIF-response genes. HIF-1α, which is overexpressed in common cancers and their metastases, plays essential roles in tumor cell adaptation to hypoxia and angiogenesis [7]. Some chemicals which enhance or inhibit this hypoxic response pathway have been reported [8]. Rotenone and diphenylene iodonium chloride (DPIC) inhibit this pathway. Rotenone is an inhibitor of the mitochondria respiratory chain. Recently, inhibition of the respiratory chain in hypoxia was shown to redistribute oxygen to non-respiratory oxygen-dependent targets, such as prolyl hydroxylase, that induce HIF-1α degradation under hypoxia [9]. In our previous study, we found that DPIC induced the degradation of HIF-1α by binding to NADPH-P450 reductase although the details of this mechanism are not clear [10]. In the current study, we added bisphenol A (BpA), an environmental endocrine-disrupting chemical, to this hypoxic pathway. EPO induction in Hep3B cells under hypoxia was completely inhibited by addition of BpA. EPO, which is a well-known hematopoietic cytokine, is found to be neuroprotective during brain ischemia in adult animal models and also to be required for normal brain development in mouse embryos, suggesting that BpA affects neural development pathway in embryos [11].

In this study, we found that BpA inhibited the induction of hypoxia-responsive proteins such as EPO via the degradation of HIF-1α. We investigated the molecular mechanism of inhibition by BpA on the hypoxic pathway. Furthermore, the essential structure of BpA for this inhibition was identified by the synthesis of BpA analogs.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin–streptomycin solution, and protease inhibitor cocktail were purchased from Sigma Chemical (St. Louis, MO). Isogen for RNA isolation was purchased from Nippon Gene (Toyama, Japan). RevertAid M-MuLV Reverse Transcriptase was purchased from MBI Fermentas (Vilnius, Lithuania). Ampli Taq Gold was purchased from Applied Biosystems (Foster City, CA). Nitrocellulose membrane, goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate, and 4-chloro-1-naphthol were purchased from Bio-Rad Laboratories (Hercules, CA). The Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA). 2,2-Bis(4-hydroxyphenyl)-propane (bisphenol A), 1,1-bis(4-hydroxyphenyl)-ethane (bisphenol E), 4,4′-methylenebisphenol (bisphenol F), and cobalt (II) chloride hexahydrate ($CoCl_2$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The Anaero Pack (oxygen absorber) was purchased from Mitsubishi Gas Chemicals (Tokyo, Japan). A proteasome inhibitor, MG132, was obtained from Peptide Institute (Osaka, Japan). Antibodies against human Hsp90 and HIF-1α were purchased from BD Biosciences (San Jose, CA) and Novus (Littleton, CO), respectively.

Synthesis of 2,2-bis(4-methoxyphenyl)propane. To a solution of bisphenol A (100 mg, 0.438 mmol) in dimethyl sulfoxide was added sodium hydride (16 mg, 0.657 mmol) under ice cooling. After the reaction mixture was stirred at room temperature for 20 min, iodomethane (40 μl, 0.657 mmol) was added at the same temperature. After the resulting mixture was stirred at room temperature for 10 min, water was added, and then the resulting mixture was extracted with diethyl ether. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by silica gel column chromatography (from 2 to 17% ethyl acetate in hexane) gave the 2,2-bis(4-methoxyphenyl)propane (BpA-OMe) (39 mg, 35%) as colorless oils.

^{1}H NMR ($CDCl_3$, 400 MHz) δ7.14 (AB-q, J = 9.0 Hz, 4H), 6.80 (AB-q, J = 9.0 Hz, 4H), 3.78 (s, 6H), 1.64 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ157.4, 143.1, 127.7, 113.2, 55.2, 41.6, 31.0; IR (NaCl neat, cm^{-1}) 2965, 2836, 1885, 1609, 1510, 1180, 1060, 829; ESI HRMS m/z calcd for $C_{17}H_{20}O_2$ $[M-H]^-$ 255.1385, found 255.1385.

Cell culture. The human hepatoma cell line Hep3B was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer of Tohoku University (Sendai, Japan). Hep3B cells were maintained in DMEM containing 10% FCS, and 1% penicillin and streptomycin at 37 °C in 5% CO_2.

Treatment. Hep3B cells were cultured in DMEM containing 10% FCS, and the FCS concentration was reduced to 0.1% at 24 h before the treatment with chemicals. For hypoxic treatment, the cells were incubated in 5% O_2, 5% CO_2, and 90% N_2 balanced with a modulator incubator chamber (Napco 7101, Winchester, VA) or were incubated in a sealed 2.5 L box with an Anero Pack for cells. Hep3B cells were incubated for 6 h under hypoxia in the presence of BpA, BpE, BpF, and BpA-OMe (50–200 μM). Hep3B cells were incubated for 6 h in the presence of BpA and BpF (200 μM) after addition of $CoCl_2$ (200 μM).

RNA isolation and reverse transcriptase-PCR. Total RNA was extracted from Hep3B cells with Isogen. A reaction mixture containing 1 μg RNA and 200 U of reverse transcriptase was reacted according to the manufacturer's protocol as follows: incubation for 10 min at 25 °C and 60 min at 42 °C, followed by heating for 10 min at 70 °C to stop the reaction. Polymerase chain reaction (PCR) was performed using a reaction mixture containing 10 pmol of each primer, 1.5 U Ampli Taq, and 100 ng cDNA according to the following protocol: 10 min at 96 °C and then 35 cycles of 30 s at 96 °C, 30 s at 56 °C, and 1 min at 72 °C. Primers for β-actin were 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense). Primers for EPO were 5′-GCCAGAGGAACTGTCCAGAG-3′ (sense) and 5′-TTCT CCAGGTCATCCTGTCC-3′ (antisense). Primers for HIF-1α were 5′-C TCAACCACAGTGCATTGTA-3′ (sense) and 5′-CAGCACTACTT CGAAGTGGC-3′ (antisense).

Immunoprecipitation and Western blotting. Cells at 80% confluence cultured under normoxia or hypoxia were lysed in 40 mM Tris–HCl buffer (pH 8.0) containing 0.2% Tween 20, 20% glycerol, 10 mM $MgCl_2$, 30 mM KCl, and MG132. Cell lysates were incubated with