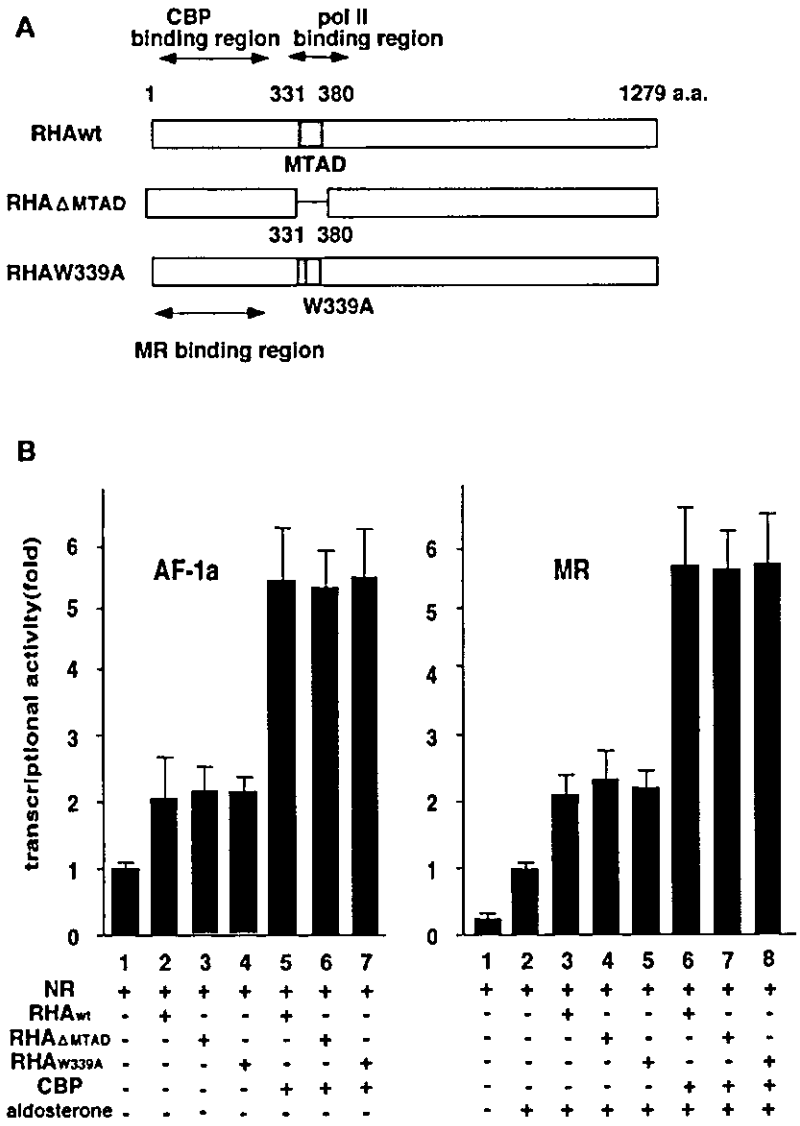

FIG. 6. RHA-RNA polymerase II binding is not necessary for potentiation of MR AF-1 by RHA/CBP complexes. (A) Schematic representation of the RHA mutants. MTAD acts as the RNA polymerase II interacting domain. However, the point mutant RHA W339A prevents this interaction. (B) RHA-RNA polymerase II binding is not necessary for potentiation of MR AF-1 by the RHA/CBP complex. 293T cells were cotransfected with 0.25 μg of a luciferase reporter plasmid bearing progesterone response elements (PREx2-tk-luc), 50 ng of an expression vector containing pc-DNA-FLAG-MR-AF-1a or pc-DNA-FLAG-MR, and 0.3 μg of either pc-DNA-HA-RHA, pc-DNA-HA-RHAΔMTAD, pc-DNA-HA-RHAW339A, or pc-DNA-CBP in the presence (+) or absence (−) of aldosterone (10^{-9} M). Bars show the fold change in luciferase activity relative to the activity of FLAG-MR-AF1a (AF-1a) or FLAG-MR (MR) in the presence of aldosterone (10^{-9} M) without transfection of coactivators. NR, nuclear receptor.

cruitment in MR (data not shown). In this study we showed that full-length MR recruited RHA/CBP complexes through the AF-1a region and that this enhanced transactivation function was ligand dependent. Considering these results together with the results of previous studies showing the different contributions of AF-1 activity to full-length MR activity induced by endogenous aldosterone and hydrocortisone (22, 26), the two different ligands appear to induce different A/B region conformations. Thus, it appears that different nuclear receptor ligands may induce different conformations not only in E/F domains but also in A/B domains (44, 51), so that ligand-selective coactivator recruitment could be a feature of both E/F and A/B domains (28).

It is thought that the specific actions of the two MR ligands observed in vivo are at least partially mediated by the restricted tissue expression of the enzyme 11βHSD2 and the subsequent inactivation of hydrocortisone in MR target tissues. While the balance between MR and glucocorticoid receptor expression levels in a given tissue or cell may lead to ligand-specific actions, these factors appear to be insufficient to fully confer ligand specificity, particularly in nonepithelial mineralocorticoid target tissues (e.g., the heart and central nervous system),

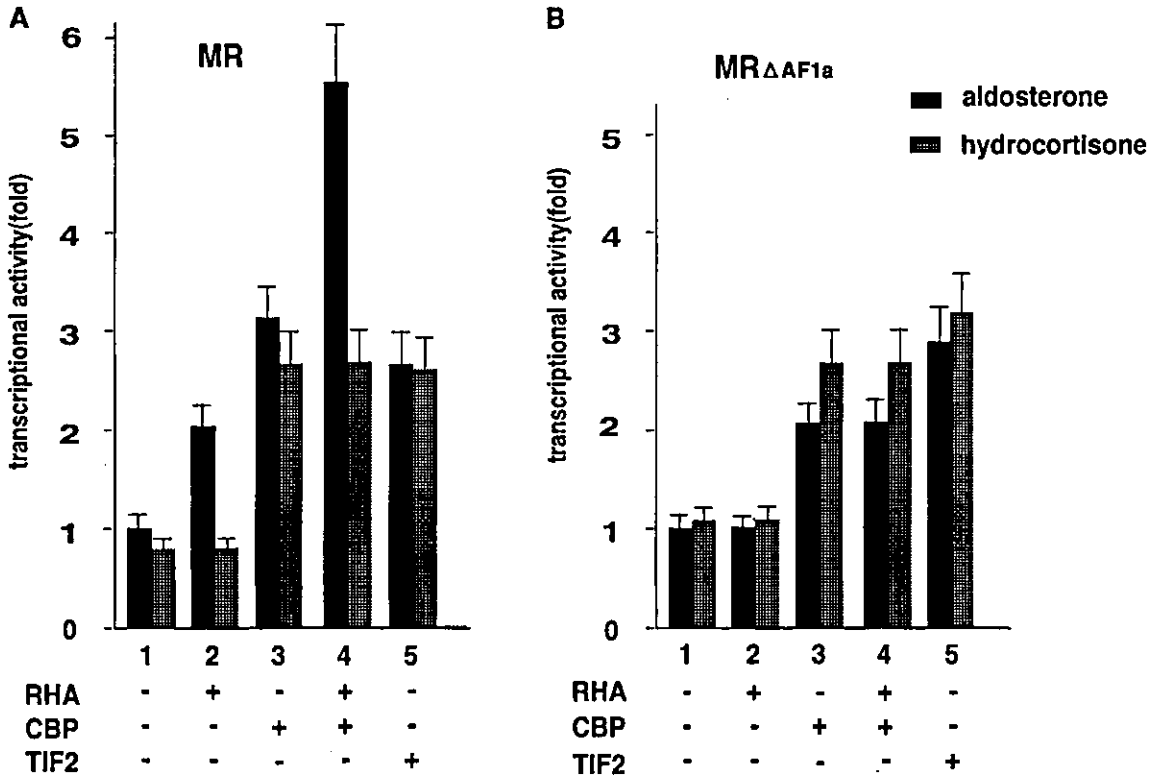

FIG. 7. RHA and CBP cooperatively potentiate MR in a ligand-selective manner. (A) Aldosterone-dependent potentiation of the MR transactivation function by the RHA/CBP protein complex was observed, but hydrocortisone-dependent potentiation was not. (B) AF-1a is essential for the ligand-selective potentiation by the RHA/CBP complex. 293T cells were cotransfected with 0.25 μg of a luciferase reporter plasmid bearing progesterone response elements (PREx2-tk-luc), 50 ng of an MR deletion mutant expression vector, and 0.3 μg of either pc-DNA-HA-RHA, pc-DNA-CBP, or pc-DNA-TIF2 in the presence (+) or absence (−) of either aldosterone (10^{-9} M) or hydrocortisone (10^{-9} M). Bars show the fold change in luciferase activity relative to the activity of FLAG-MR-FL (MR) or FLAG-MRΔAF1a (MRΔAF1a) in the presence of aldosterone (10^{-9} M) without transfection of coactivators.

where 11βHSD2 is not expressed (33). We show here that the recruitment of RHA/CBP coactivator complexes may discriminate between the actions of the two endogenous MR ligands. The tissue-specific activity of the MR ligands suggests the possible existence of tissue-specific components of coactivator complexes (57, 59, 60). A recent report using MR knockout mice shows that MR is essential for the neurogenesis of granule cells in the hippocampus (17). As the ratio between serum aldosterone and hydrocortisone levels varies during neuronal development, it is thought that MR functions at a critical stage during neuronal development. Therefore, to better understand MR ligand-specific activity at the molecular level, it will be of great interest to assess the expression and physiological function of the RHA/CBP complex in different tissues.

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Wnt/β-Catenin and Estrogen Signaling Converge *in Vivo**

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Wnt and estrogen signaling represent important regulatory pathways, each controlling a wide range of biological processes. While an increasing number of observations suggest potential convergence between these pathways, no direct evidence of their functional interaction has been reported. Using human colon and breast cancer cells, we found that estrogen receptor (ER) α- and β-catenin precipitated within the same immunocomplexes, reciprocally enhanced the transactivation of cognate reporter genes, and were reciprocally recruited to cognate response elements in the promoters of endogenous target genes. Using transgenic *Drosophila* that ectopically expressed human ERα alone or together with metabolically stable β-catenin/Armadillo mutants, we demonstrated genetic interaction between these signal transducers *in vivo*. Thus, we present here the first direct evidence of cross-talk between Wnt and estrogen signaling pathways via functional interaction between β-catenin and ERα.

Estrogens regulate a plethora of physiological functions in the developing and adult organism and act predominantly via the activation of ERα[1] and ERβ. Liganded ER dimers bind to promoter estrogen response elements (EREs) and regulate the transcription of target genes. This ER-mediated regulation requires the recruitment of different co-factor complexes and is associated with rearrangement of chromatin structure at EREs within target gene promoters (1, 2). ER can also act as a co-factor at non-ERE sites via interaction with other DNA-bound transcriptional factor complexes, such as c-Jun/c-Fos on the AP-1 site (3) or c-Jun/NFκB on the tumor necrosis factor response element (4). The physiological significance of ERs is demonstrated by the severe abnormalities in development and function of major organs and tissues in mice with ablated ERα and/or ERβ (5). Also, both positive and negative impacts of estrogens in different types of cancer have been well documented (6).

Wnt signaling plays a critical role in numerous processes of development and in adult tissues and appears to be conserved across all animal taxa. β-Catenin is an intracellular transducer of canonical Wnt or Wnt/β-catenin signaling and, thus, has a dual function: as a transcriptional factor and, in a cadherin-bound form, as a regulator of cell adhesion and migration. Cytoplasmic or signaling β-catenin is unstable and rapidly targeted to phosphorylation-ubiquitination-coupled proteasomal degradation. Wnt signaling inhibits this degradation, resulting in the accumulation of β-catenin in the nucleus and its association with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcriptional factors that leads to the activation of Wnt target genes. Mutations that increase the stability of cytoplasmic β-catenin have been implicated in numerous malignant transformations and represent a leading cause of colorectal tumorigenesis (7–9).

Consistent with the concept of morphogen gradients (10) β-catenin exerts different biological effects, such as induction of cell proliferation and apoptosis or stimulation and repression of the same target genes, in a threshold-dependent manner (11, 12). Thus, slight modulation of β-catenin signaling through cross-talk with other pathways may trigger serious physiological consequences. Potential cross-talk between Wnt/β-catenin and estrogen signaling *in vivo* has been implicated in physiological studies on tissues as different as brain (13) and uterus (14). Furthermore, although males and females develop colorectal cancer with approximately the same frequency, its incidence rate is significantly lower in women undergoing hormone replacement therapy (15, 16). While these and other observations suggested the possibility of functional interaction between ER and β-catenin, previous attempts failed to detect such an interaction (13, 17, 18), and no direct evidence of Wnt and estrogen signaling pathway convergence has been reported.

Compared with vertebrates, Wnt signaling has been far better characterized in *Drosophila*, in which it is not obscured by involvement of other, evolutionary more recent multiple pathways. Thus, *Drosophila* provides a powerful experimental system for analysis of functional interaction *in vivo* between Wnt signaling and other regulatory pathways, including those immerged at the later stages of evolution. Therefore, in addition to mammalian cells, to detect functional interaction between Wnt/β-catenin and estrogen signaling *in vivo* we used transgenic *Drosophila* that ectopically expressed human ERα coupled to an ERE-dependent green fluorescent protein (GFP) reporter gene alone or together with constitutively active mutants of Armadillo, a *Drosophila* homologue of β-catenin. Using different approaches, we obtained in this study the first evidence of physical association and transcriptional and genetic interaction *in vivo* between ERα and β-catenin.

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; TCF, T cell factor; LEF, lymphoid enhancer factor; TBE, TCF/LEF binding element; CSFCS, charcoal-stripped fetal calf serum; ChIP, chromatin immunoprecipitation; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; LBD, ligand binding domain; wt, wild-type.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and Immunoblotting—Cells grown in the presence of charcoal-stripped fetal calf serum (CSFCS) were transfected with FLAG-hERα expression vector and harvested 28–30 h post-transfection, after treatment for 3 h with vehicle (ethanol) or 10^{-8} M 17β-estradiol (Sigma), tamoxifen (Sigma), or ICI 182,780 (Tocris). Anti-β-catenin E-5 or H-102 antibodies (Santa Cruz Biotechnology) or preimmune rabbit serum IgG (as a negative control) were used for immunoprecipitation. Western blots were visualized with anti-FLAG M2 (Sigma) or anti-ERα HC-20 (Santa Cruz Biotechnology) antibodies.

Transfection and Reporter Assay—Cells grown in Opti-MEM, 5% CSFCS were transfected with 250 ng of reporter (ERE-tk-luc or tk-luc for MCF7 cells and TOPFLASH or FOPFLASH for colon cancer cells) and 1 ng of pRl (Promega) plasmid (control for transfection efficiency) together with 100 ng of empty (control) or cDNA (β-catenin S33Y for MCF7 cells and ERα for colon cancer cells) expression vector and treated for 16–20 h with vehicle or 10^{-8} M ligand, as indicated. To nullify nonspecific effects on basal promoters, TOPFLASH and ERE-tk-luc reporter activities were normalized against FOPFLASH and tk-Luc reporter activities, respectively, from parallel experiments.

Chromatin Immunoprecipitation (ChIP) Assay—Association of ERα and β-catenin with ERE in the pS2 gene promoter (19) and TCF/LEF binding element (TBE) in the Axin2 gene promoter (20) was analyzed using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) and HC-20 or E-5 antibody, respectively. As a control for nonspecific chromatin precipitation with these antibodies, a set of primers was used to amplify a pS2 gene DNA segment that does not have ERE or TBE sequences. In addition, IgG from normal preimmune rabbit serum was used as a negative control.

Histology and Immunostaining—All techniques were performed as described previously (21, 22). Expression of ERα and GFP in *Drosophila* eye discs were detected using Zeiss Confocal Laser Scanning System 510 and quantified by calculation of pixels of the corresponding signals using Adobe Photoshop 7 software facilities. TUNEL labeling was performed using the TACS2 TdT-Fluor *In Situ* Apoptosis Detection Kit (Trevigen).

Drosophila Lines and Stocks—The *UAS-ΔArm* and *UAS-ArmS10* mutants were obtained from the Bloomington *Drosophila* Stock Center. Generation and characterization of the used *UAS-ERα*, *ERE-GFP* transgenic *Drosophila* lines were described in Ref. 23. Briefly, cDNA encoding full-length human ERα, ligand binding domain (LBD) deletion mutant ERα-(1–302), or GFP reporter under control an ERE containing promoter were recloned into the pCaSpeR vector. Transgene constructs together with pπ25.7wc transposase were microinjected into w^{1118} embryos using a micromanipulator (Leica). Several independent transformant lines have been generated. To target ERα expression into the eye disc, transgenic *Drosophila* were crossed with flies of a *GMR-GAL4* line expressing GAL4 driver in the retina under control of the tissue-specific glass multimer gene promoter.

RESULTS

Physical Association of ERα and β-Catenin—Human colon cancer HCT116 cells express metabolically stable β-catenin due to mutation at its putative phosphorylation site. These cells, however, do not express detectable ER. HCT116 cells were transfected with a FLAG-tagged human ERα expression plasmid, and endogenous β-catenin was immunoprecipitated from cell lysates following 3-h preincubation with estrogen or vehicle. IgG from normal rabbit serum was used as a control for nonspecific immunoprecipitation. Obtained immunocomplexes were subjected to Western blotting and analyzed by immunostaining with antibodies against FLAG-tag and ERα.

ERα co-immunoprecipitated with β-catenin even in the absence of ligand; however, ERα-β-catenin association was markedly stimulated by estrogen (Fig. 1*A*). Similar results (data not shown) were obtained using SW480 human colon cancer cells, in which non-mutant β-catenin was stabilized by a loss-of-function mutation in the gene of tumor suppressor Adenomatous polyposis coli, an essential component of the β-catenin degradation machinery. Brief exposure to ligand did not affect FLAG-ERα expression in this (Fig. 1*A*) or further experiments.

As anti-β-catenin antibodies co-precipitated a C-terminally truncated FLAG-ERα-(1–396) (Fig. 1*B*), it appeared that an intact LBD was not essential for the ER interaction with β-catenin. Predictably, C-terminal truncation of ERα abolished the ligand sensitivity of the interaction.

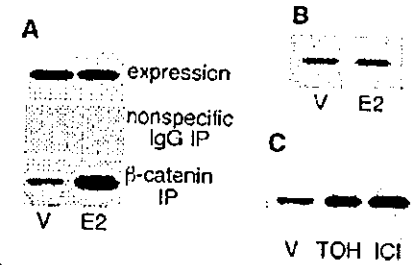

FIG. 1. **Association between ERα and β-catenin in mammalian cells.** *A–C*, anti-FLAG immunostaining of Western blots of immunocomplexes precipitated with antibodies against β-catenin or with nonspecific rabbit IgG (*A*) from HCT116 cells expressing FLAG-tagged full-length ERα (*A*, *C*) or C-terminally truncated ERα (*B*) pretreated with vehicle (*V*), estradiol (*E2*), tamoxifen (*TOH*), or ICI 182,780 (*ICI*), as indicated. *IP*, immunoprecipitation.

We then analyzed whether ligands that inhibited the transcriptional activity of ERα would also affect its interaction with β-catenin. Immunoprecipitation of ERα with antibodies against β-catenin was significantly stimulated by the ERα partial, tamoxifen, and complete, ICI 182,780, antagonists (Fig. 1*C*).

Transcriptional Interaction between ERα and β-Catenin—Next, we investigated whether the apparent physical association between ERα and β-catenin was consequential for transcriptional function of the proteins. Transactivation of an ERE-dependent reporter by endogenous ER was studied in human breast cancer MCF7 cells, in which the Wnt pathway is practically silent. Expression of stabilized β-catenin S33Y in these cells enhanced ligand-dependent expression of the reporter without affecting its basal activity in the absence of ligand (Fig. 2*A*). Expression of ERα in human colon cancer SW480 (Fig. 2B) and HCT116 (data not shown) cells enhanced the activation of the Wnt-responsive TOPFLASH reporter by endogenous β-catenin in the absence of ligand. Treatment with estrogen resulted in further moderate activation of reporter expression, while ER antagonists appeared not to affect reporter gene activity (Fig. 2*B*).

The reciprocal activation of cognate reporters in the transfection experiments suggested that ERα and β-catenin might reciprocally recruit each other to their corresponding response elements in endogenous target gene promoters. Indeed, antibody against β-catenin precipitated ERE of the pS2 gene promoter from chromatin of β-catenin S33Y expressing MCF7 cells in an estrogen-dependent manner (Fig. 2*C*). Conversely, anti-ERα antibody precipitated in a ligand-dependent manner Axin2 gene promoter putative TBE from chromatin of SW480 cells transfected with an ERα expression construct, while recruitment of β-catenin to the TBE was not sensitive to the presence of estrogen (Fig. 2*D*). The used antibodies did not display nonspecific chromatin precipitation (Fig. 2*E*).

Consistent with the results obtained using MCF7 cells, ERα transactivation was markedly enhanced *in vivo* by the stabilized Armadillo mutants ΔArm (24) (Fig. 2*F*) or ArmS10 (25) (data not shown) when ectopically co-expressed in the *Drosophila* eye disc.

Genetic Interaction between ERα and β-Catenin—Constitutive activation of Armadillo in the *Drosophila* eye disc has been shown to induce apoptosis and consequent degeneration in the adult eye (26, 27). Potentiation of β-catenin transcriptional activity by ERα in SW480 cells (Fig. 2*B*) and functional interaction between ERα and Armadillo (Fig. 2*F*) would predict activation of endogenous Armadillo by the ectopic ERα expression in

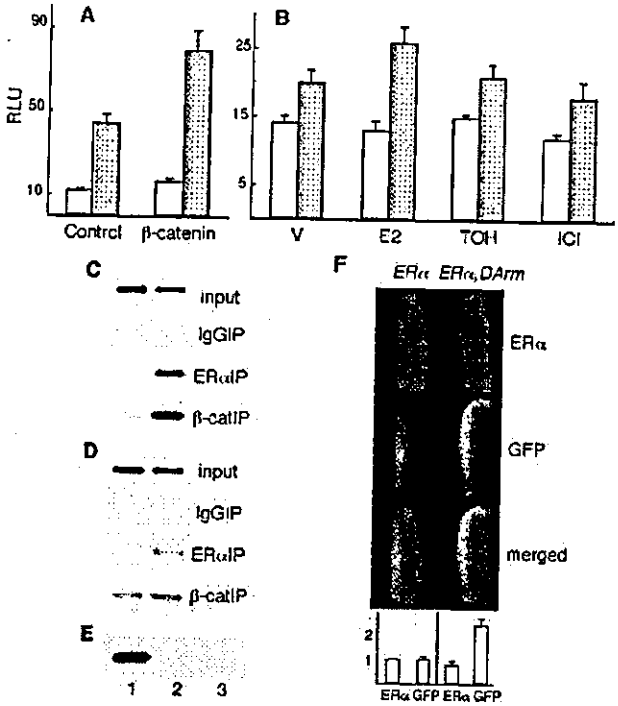

FIG. 2. **Transcriptional interaction between ERα and β-catenin.** *A*, MCF7 cells were transfected with ERE-tk-Luc reporter construct together with empty (*Control*) or β-catenin S33Y expression vector and treated with vehicle or estradiol (*open* and *filled bars*, respectively). *B*, SW480 cells were co-transfected with Wnt/β-catenin-responsive reporter and empty or ERα expression vector (*open* and *filled bars*, respectively) and treated with vehicle (*V*), estradiol (*E2*), tamoxifen (*TOH*), or ICI 182,780 (ICI), as indicated. The data represent the mean ± S.D. of three independent experiments. *C* and *D*, ChIP assay of the putative ERE of the pS2 gene promoter in MCF7 cells (*C*) and the putative TBE of the Axin2 gene promoter in SW480 cells (*D*) with anti-ERα (*ERαIP*) or β-catenin (*β-catIP*) antibodies or preimmune rabbit IgG (*IgGIP*) as a negative control. Cells were pretreated for 3 h with vehicle (*left column*) or estradiol (*right column*). *E*, control for a nonspecific chromatin immunoprecipitation: amplification of a pS2 gene DNA segment that does not contain ERE or TBE sequences from DNA samples used for PCR presented in C, *right column*: input (*lane 1*), ChIP with anti-ERα (*lane 2*), or anti-β-catenin (*lane 3*) antibodies. The data shown are representative of typical results of at least three independent ChIP experiments. *F*, estrogen-induced expression of an ERE-dependent GFP reporter (*green*) in *Drosophila* third instar larva eye discs ectopically expressing human ERα (*red*) alone or together with constitutively active Armadillo mutant ΔArm. Similar results were obtained with a different constitutively active Armadillo mutant, ArmS10.

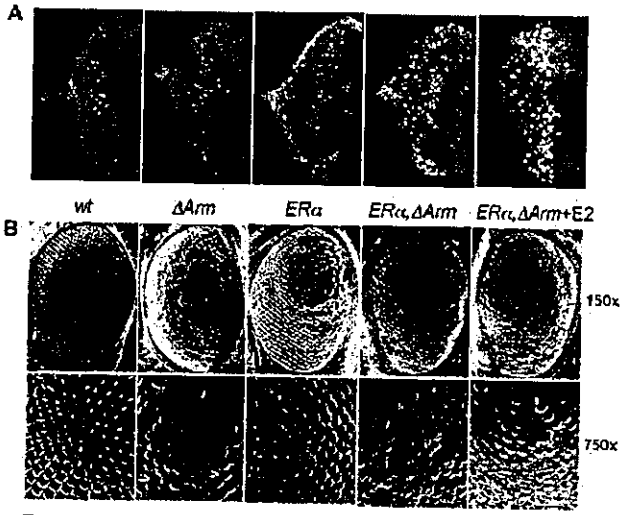

FIG. 3. **Genetic interaction between ERα and β-catenin/Armadillo in *Drosophila*.** *A*, apoptosis (*green*, fluorescein isothiocyanate TUNEL-labeled cells) in third instar larval eye discs; *B*, scanning electron microscope images of adult eye from *Drosophila* with ectopic expression of ERα, stabilized Armadillo mutant ΔArm, or both, as indicated. The same phenotypes were produced in experiments using other independently obtained *Drosophila* lines with different chromosomal localization of the ERα transgene.

the *Drosophila* eye disc leading to development of a phenotype characteristic of abnormal Wnt/β-catenin activation.

We performed TUNEL staining of the third instar larval eye discs with ectopic expression of ERα alone or together with the constitutively active Armadillo mutant ΔArm. When expressed singly, ERα and ΔArm both induced a slight increase in apoptosis compared with wild-type (wt) eye discs from *Drosophila* of the parental line. Co-expression of ERα and ΔArm resulted in a marked increase in apoptotic cell number. Importantly, while estrogen had no discernible effect on apoptosis in wt eye discs and those expressing either ERα or ΔArm alone (data not shown), treatment with estradiol significantly increased apoptosis rates when ERα and ΔArm (*ERα,ΔArm*+E2) were co-expressed (Fig. 3*A*). Activated Armadillo has a mild apoptotic effect in the third instar larva eye disc, reportedly due to the protective counteraction at this stage by the EGFR/MAPK signaling (27). This allowed us to detect differences in apoptosis patterns in transgenic fly eye discs at this developmental stage that would otherwise be difficult to distinguish due to the onset of massive cell death at the later stages.

We compared adult eye phenotypes of flies from these transgenic lines and the wt (Fig. 3*B*). The normal *Drosophila* eye is composed by regularly spaced ommatidia with regularly oriented interommatidial bristles. Expression of ERα in the eye disc leads to development of phenotypes similar to those caused by expression of ΔArm: rough eye appearance and disorientation or loss of interommatidial bristles. Co-expression of ΔArm and ERα synergistically enhanced this abnormal eye development. Again, while estradiol appeared not to affect the separate ΔArm or ERα expression phenotypes (data not shown), treatment with estrogen, however, further aggravated the severity of eye abnormalities in the ERα and ΔArm co-expression mutants (*ERα,ΔArm*+E2). Consistent with β-catenin-ERα-(1–396) co-immunoprecipitation, ectopic expression of the LBD deletion mutant ERα-(1–302) in the eye disc produced a phenotype closely resembling that with the full-length ERα (data not shown).

DISCUSSION

We found that β-catenin associated with ERα even in the absence of ligand and that estrogens further enhanced this interaction. While it is possible that the ligand-independent association was due, at least in part, to the overexpression of one of the interacting proteins, the association between β-catenin and C-terminally truncated ERα suggested that the ligand binding was not essential but might rather induce a more favorable conformation for ERα to interact with β-catenin. This may be of functional significance at physiological concentrations of the interacting proteins. Interestingly, β-catenin recruitment to EREs and ERα recruitment to TBEs in the promoters of endogenous target genes were both highly ligand-dependent. The apparently equal stimulation of ERα-β-catenin interaction by ER agonists and antagonists may have important implications for the design of novel therapeutic strategies.

Our most significant finding was that ERα functionally interacted with β-catenin/Armadillo *in vivo* in transgenic *Drosophila*. The ligand-dependent transactivation function of ERα was significantly enhanced by the co-expression of stabilized Armadillo mutants. Abnormalities in the eye development in-

duced by targeted expression of activated Armadillo and ERα were of a similar nature. Co-expression of both proteins synergistically enhanced the abnormal phenotype that was further aggravated by treatment with estradiol. Importantly, in mammals, estradiol is shown to have a prominent neuroprotective activity thought to be mediated by ER (28).

Physical and transcriptional interaction between β-catenin and androgen receptor has been observed previously (17, 18). However, in experiments presented in these reports no interaction between β-catenin and other nuclear hormone receptors, including ER, has been detected.

Thus, we have shown that Wnt and estrogen signaling pathways cross-talk *in vivo* through functional interaction between ERα and β-catenin. This interaction may underlie mechanisms of estrogen effects in pathological conditions and processes in which abnormalities of Wnt/β-catenin signaling have been implicated, such as in colorectal cancer. In addition, we have established a novel experimental system in which to identify factors conserved between humans and *Drosophila* that may be involved in regulation of cross-talk between Wnt and estrogen signaling and for the screening of novel compounds able to interfere with this cross-talk.

Although other mechanisms may be involved (*e.g.* intranuclear sequestration), transcriptional modulation appears to be the major mechanism of functional ERα-β-catenin interaction. The genomic function of nuclear receptors is dependent on the recruitment of different coactivator and chromatin remodeling complexes (1, 2, 29, 30). β-Catenin has been shown to recruit coactivators, such as the p300/CBP complex (31), and components of the mammalian SWI/SNF and RSC chromatin remodeling complexes (32) that are also known to interact with ERα. Recruitment of additional co-activator and chromatin remodeling complexes may account for the transcriptional outcome of ERα-β-catenin interaction. The physiological consequences of this interaction may also depend on cell and tissue specificity in composition of the recruited regulatory complexes. Further experiments to identify all ERα-β-catenin complex components are required to determine whether the ERα-β-catenin interaction results only in quantitative changes in the composition of the recruited regulatory proteins or if factors specific to ERα-β-catenin protein complexes are involved.

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Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats

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Abstract

There is an endocrinological concern that environmental endocrine disrupters (EEDs) may influence sexual differentiation. Bisphenol A (BPA), one of EEDs, is released from polycarbonate plastics, and has been detected in the human umbilical cord. In this study, we examined the effect of BPA on the sexual differentiation of open-field behavior and the sexually dimorphic nuclei in the brain in the offspring of rats exposed to BPA during the fetal and suckling periods at a dosage below the human tolerable daily intake (TDI) level. In the control group, females were more active in the open field and had a larger locus coeruleus (LC) volume than males. BPA abolished and inverted the sex differences of the open-field behavior and the LC volume, respectively, without affecting the reproductive system. We also compared the effects of estrogenic compounds, diethylstilbestrol (DES) and resveratrol (RVT), to that of BPA because of their structural similarities. DES affected the open-field behavior, LC volume and reproductive system, while RVT affected the LC volume and the reproductive system. These results suggest that the brain is highly sensitive to BPA at a dosage below TDI and that the disrupting effects of BPA on sexual differentiation may vary from those of RVT and DES.

Keywords: Bisphenol A; Resveratrol; Diethylstilbestrol; Sexual development; Sexual differentiation; Reproduction; Locus coeruleus

1. Introduction

A great endocrinological concern has recently arisen that certain industrial chemical substances, which are released into our surrounding environment, may influence the central nervous system (CNS) as well as the reproductive system in many animals (Colborn et al., 1993). These chemicals are called 'environmental endocrine disrupters (EEDs)'. Bisphenol A (BPA) is one of the most famous EEDs and it is released from polycarbonate plastics, epoxy resins of food cans etc. (Krishnan et al., 1993; Society of the Plastics Industry, 1996). Human fetuses have also been reported to be contaminated with BPA (Sakurai and Mori, 2000; Brock et al., 2001). BPA binds to both the estrogen receptor α (ERα) and β (ERβ), and BPA induces a proliferation of estrogen receptor expressing MCF-7 cells in an estrogen-dependent manner (Krishnan et al., 1993; Sohoni and Sumpter, 1998; Hiroi et al., 1999). In addition, BPA has been found to increase both the weight of the uterus and the release of prolactin into the blood in female rodents, similar to estrogen (Steinmetz et al., 1997; Ashby and Tinwell, 1998; Steinmetz et al., 1998). These results indicate that BPA has a common property with estrogenic substances.

The gonadal steroids differentiate neurons and then create the sexual dimorphisms of the brain structure and behavior (MacLusky and Naftolin, 1981; Kelly et al., 1999). Up to now, there is little information about the effect of BPA on the CNS, especially regarding sexual

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differentiation of the brain and behavior. In a previous study, we found that the BPA exposure during the fetal and suckling periods at a dosage (1.5 mg/kg per day) below the no-observed-adverse-effect level (NOAEL) disrupted the sex difference in open-field behavior and moreover the gender difference of the size of the locus coeruleus (LC), but BPA did not affect the reproductive function or the size of the sexually dimorphic nucleus of the preoptic area (SDN-POA; Kubo et al., 2001). The NOAEL of BPA is determined as 50 mg/kg per day by various in vivo toxicity studies including a reproductive toxicity test. On the basis of the NOAEL, the resolution limitation is calculated as 2.5 mg/l (ppm), and the tolerable daily intake (TDI), which is the maximum acceptable dose in humans, is also calculated as 50 μg/kg per day (from the web site: www: http://www.bisphenol-a.org/health/exposure/consumer/research.html).

For the risk assessment of BPA, it is important to confirm that BPA at or below the TDI does not cause any adverse effects. The main purpose of the present study is to elucidate whether the exposure of very low dose of BPA during the fetal and suckling periods also affects non-reproductive sexual differentiation of both the open-field behavior and the LC structure, and the reproductive function including sexual behavior. Two concentrations of BPA were set at lower levels than that used in our previous study, and the daily intake of BPA was under the TDI in the lower BPA group. An additional purpose of the study is to compare these effects of BPA to those of other estrogenic compounds. In this study, we used two estrogenic chemicals showing structural similarity to BPA. One is *trans*-resveratrol (RVT) which is a phytoestrogen found in grapes, red wine, peanuts, and other fruits. RVT has an estrogenic potential (Gehm et al., 1997), however, there is little information about the effect of RVT on sexual differentiation of the CNS. The other is diethylstilbestrol (DES) which is a synthetic estrogen, and the estrogenic potential of DES is similar to that of estradiol. DES is often used as an estrogenic control to study the effect of EEDs, because the effects of DES have already been reported regarding sexual differentiation of both the CNS and the reproductive system (MacLusky and Naftolin, 1981; Döhler et al., 1984; Csaba et al., 1986; Tarttelin and Gorski, 1988; Sharpe et al., 1995; Ashby et al., 1997; Vancutsem and Roessler, 1997; Kwon et al., 2000; Atanassova et al., 2000).

2. Materials and methods

2.1. Animals and treatments

Female and male Wistar rats at 11 weeks of age were purchased from Kyudo Corp. in Japan and then were housed in standard cages for 2 weeks. After copulation at 13 weeks of age, five dams were exposed to vehicle as control (CON group), six dams to BPA at a dose of 0.1 mg/l (lower BPA-treated group; BPL), six dams to BPA at a dose of 1 mg/l (higher BPA-treated group; BPH), five dams to *trans*-RVT at a dose of 5 mg/l, and five dams to DES at a dose of 50 μg/l, respectively. All estrogenic compounds were purchased from Sigma (St. Louis, MO, USA), and dissolved in 0.01% ethanol in distilled water. The RVT dose was determined based on the normal contents usually found in red wine (Goldberg et al., 1996; Sato et al., 1997), and the dose of DES was also determined based on the findings of another previous study (Ashby et al., 1997). All of dams were allowed to feed and drink ad libitum from the day that sperm was detected in a vaginal smear in the morning, until their pups were weaned on postnatal day (PND) 21. All of the experiments were performed under the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Government of Japan.

The litters were standardized on PND 1 (the day after birth) to both five males and five females by removing any excess offspring after the evaluation of sexual development of all pups as below. All offspring were housed in same-sex and same-litter groups after weaning and were allowed food and tap water ad libitum. As a result, we administered approximately 30 and 300 μg BPA per kg body weight per day, 1500 μg RVT per kg body weight per day, and 6.5 μg DES per kg body weight per day only to mother rats in the BPL, BPH, RVT and DES groups, respectively. It is important that the daily intake of BPA in the group exposed to lower BPA was observed to be less than the TDI.

2.2. Evaluations of sexual development

We evaluated the sexual development of offspring using previously described methods (Omura et al., 2000; Ogata et al., 2001). Briefly, on PND 1, the body weight and anogenital distance (AGD) of all newborn pups were measured. The AGD was defined as the distance between the center of the anus and the posterior edge of the genital tubercle when the skin area was naturally extended without stretching, and the AGD was expressed by the absolute AGD divided by the cube root of body weight ($mm/g^{1/3}$) in order to remove the influence of the body size. The AGD was measured using a micrometer by a single experimenter who was blinded to the treatment groups. Starting on PND 17, the male pups were examined daily to check for testicular descent. Starting on PND 24, the female rats in DES-treated group were examined daily for vaginal opening, while starting on PND 29, other female offspring were examined daily for vaginal opening.

2.3. Open field test

When the offspring became 6 weeks old, the open field test was performed to evaluate novelty response. The open field apparatus was a square field (78 × 56 × 57.5 cm), in which a box (17.5 × 9.5 × 8 cm) was placed at the center. Each rat was placed along one side in the apparatus at the beginning of the test, and was allowed to move freely for 10 min. The total distance that the animal moved and the time that the rat spent in the center area were automatically analyzed with a computer-based videotracking system (Ethovision v1.96, Noldus Info. Tech., Netherlands), and the number of rearing (rats that stood up on their hindlegs) was manually recorded. The open field was cleaned between each subject to prevent olfactory cues from affecting the behavior of subsequently tested rats. This experiment was conducted between 13:00 and 18:00 h and each animal was tested only once.

2.4. Sexual behavior

We tested the sexual behaviors of male and female rats at 11–12 weeks of age which were conducted after 19:00 h. In the test of male sexual behavior, each male was allowed 30 min to habituate to an experimental cage, the day before the experiment. On the experimental day, a male rat was introduced in the experimental cage 1 min before the introduction of a stimulus female. The stimulus females were ovariectomized, and sexual receptivity had been induced by the subcutaneous injection of 10 μg estradiol benzoate (Sigma) in 0.1 ml corn oil and 500 μg progesterone (Sigma) in 0.1 ml corn oil, 48 and 6 h prior to testing, respectively. The following parameters of male sexual behavior were recorded: number of mounts (number of mounts with pelvic thrusting with and without vaginal penetration that preceded ejaculation), number of intromissions (number of mounts with vaginal penetration that preceded ejaculation), intromission rate (= (number of intromissions/number of mounts) × 100%), latency to ejaculation (time from the first intromission to ejaculation), duration from first mount to first ejaculation, and postejaculatory interval (time between ejaculation and the first mount of the next ejaculatory series). The numbers of mounts and intromissions were calculated per 10 min to evaluate accurately, because their numbers depended on the duration from first mount to first ejaculation. All males were observed for one complete ejaculatory series and the subsequent postejaculatory interval and were removed from the testing cage.

On the other hand, we counted the number of ear wiggles and calculated the lordosis quotient (LQ = (number of lordosis/ten mounts) × 100%) for the evaluation of proceptivity and receptivity in female rats, respectively. In addition, we recorded the rejection score as previously described (Meston et al., 1996): Briefly, a score of 0, 1, 2 and 3 was given to rats who showed no rejection, displayed vocal rejection, kicked with vocalization, and showed maximum rejection, respectively. Each female was placed with a sexually vigorous male rat in an experimental cage for 10 min and then was removed from that cage. The lordosis response of each female was scored quantitatively by recording the presence or absence of lordosis in response to a mount with pelvic thrusting by the male.

2.5. Measurements of reproductive organ weights, sperm counts and serum hormone levels

The estrus cycle was evaluated in female animals as previously described (Kwon et al., 2000; Ogata et al., 2001). Each male rat at 12 weeks of age was killed by carbon dioxide at necropsy, and also each female rat at the same age was killed at the proestrus stage on the day after the diestrus day by the same procedure. The reproductive organs were weighed just after being dissected as previously described (Omura et al., 2000; Ogata et al., 2001). The testis and the cauda epididymis were homogenized in STM solution (8.75 g NaCl and 0.5 ml Triton X-100 were dissolved in distilled water for 1 l of STM solution) with a Waring blender. After that, homogenization-resistant spermatids or sperm were counted using hemocytometer. The other testis was fixed in Bouin's solution, embedded in paraffin wax, thinly sectioned and stained with periodic acid Schiff reagent (PAS) and hematoxylin. In the histopathologic examination, seminiferous tubules were classified into 14 stages. All cross-sections of seminiferous tubule in one transverse section of the testis were examined and the histopathologic changes were evaluated. The degeneration of one or two germ cells in one seminiferous tubule was not regarded as a histopathologic change. The ovary was fixed in 10% neutral buffered formalin solution, embedded in paraffin, thinly sectioned, and stained with hematoxylin and eosin. Five random sections of the ovary were examined for normal follicles (primordial, antral and growing follicles) and corpora lutea and follicular atresia. A histopathologic examination was carried out without any knowledge regarding the treatment.

At necropsy, blood samples were collected from the posterior vena cava, and serum was separated and stored at −80 °C until assays. The serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, testosterone, and 17β-estradiol were measured by radioimmunoassay. The test kits used were the Rat LH [125I] assay system (Amersham Pharmacia Biotech, Japan), FSH [125I] assay system (Amersham Pharmacia Biotech), Rat Prolactin [125I] assay system (Amersham Pharmacia Biotech), Coat-A-Count® Total Testosterone kit (Iatron Laboratories,

Inc., Japan), and Double Antibody Estradiol kit (Iatron Laboratories, Inc.). For each assay, all samples were randomized prior to analysis.

2.6. Measurements of sexually dimorphic nuclei

At 14 weeks of age, the rats were anesthetized with ether and perfused transcardially with 10% formalin. Next, the brains were removed, weighed and soaked in 20% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 2 days. Serial frontal sections of the frozen brain were cut at 40 μm intervals through the entire SDN-POA and LC using a frozen microtome. All sections were stained with a 0.1% solution of Cresyle violet (Merck) brought to pH 4.0 with acetic acid. Both a NIKON microscope and a digital camera (PDMC1, Polaroid) linked to an imaging system were used to capture and digitize the Nissl-stained sections (NIH IMAGE software). The SDN-POA and LC nuclear outlines were defined by tracing with a mouse, respectively. Throughout the entire of SDN-POA and LC, the total area (μm^2) was determined for each section. The total volumes of the SDN-POA and the LC were then calculated by multiplying the total areas of each section by the average thickness of the sections, according to a previously described method (Gorski et al., 1978; Guillamón et al., 1988). The number of neurons was counted three times using 140 μm interval grids under × 200 microscopic magnification, and the total number of neurons was estimated by summing up the counted cells.

2.7. Statistics

This study was divided into three blocks in which each experiment was replicated three times, because of the large number of animals involved. All data were collected after the third block finished, and then were analyzed using the STATVIEW 5.0J software for Windows (SAS Inc). The offspring data during the suckling period were analyzed using the litter as a unit, and the data after weaning were analyzed using each animal as a unit. When we analyzed the data for the open field test and those regarding the brain structure, we used one-way ANOVA for each sex as well as Student's *t*-test between males and females because our focus was to compare any sex differences based on sex. The data for sexual development, the reproductive organs including sperm, and serum hormone levels were analyzed by one-way ANOVA followed by Fisher's PLSD test for each sex. Statistical differences were considered significant when the *P*-value was below 0.05. All results are presented as the means and standard error (S.E.M.).

3. Results

3.1. Sexual development

The endopoints regarding the sexual development of the offsprings are shown in Table 1. None of the chemicals induced a change in body weight on PND 1 or AGD in either the male or female offspring. BPA exposure did not affect the sexual development in either the male and female offspring. BPA did not affect the day of testicular descent in the male offspring, or that of vaginal opening in the female offspring. In addition, RVT exposure did not affect male sexual development, while RVT delayed the day of vaginal opening in females and their body weights on the day of vaginal opening increased in comparison to the control females. In contrast to BPA and RVT, DES influenced female sexual development but not that in males. The day of vaginal opening was accelerated and the body weight on the day of vaginal opening decreased in DES-exposed females.

3.2. Open-field behavior

In the open field test, we measured the total distance moved to evaluate the locomotor activity, and also measured the number of rearings and the length of time that each rat stayed in the center zone to evaluate the exploratory behavior (Fig. 1). All three estrogenic chemicals affected the open field behavior in different manners. Females moved significantly longer, reared more frequently, and stayed longer in the center area than males in the control group as well as in the RVT group. All of these sex differences, however, disappeared in the BPH and DES groups, whereas the sex difference in the exploratory behavior but not in locomotion also disappeared in the BPL group. As shown in the figures, the disappearance of sex differences regarding both locomotion and rearing behavior in the BPA and DES groups appeared to be due to the fact that the male levels gradually began to approach the female levels (demasculinization of males). The disappearance of the sex difference regarding the length of stay in the center area seemed to be due to the fact that the rats appeared to reach a neutral level between that of males and females (demasculinization of males as well as defeminization of females) in both BPA-treated groups, whereas both males and females in the DES group stayed longer in the center zone than the control males and females, respectively.

3.3. Sexual behavior

In male rats, three estrogenic chemicals differentially affected the sexual behavior. The number of mounts in the BPA, RVT and DES groups did not differ from that

Table 1
Effect on the sexual development

	Control	BPL	BPH	RVT	DES
Male					
Body weight on PND1 (g)	5.91±0.05 (5)	6.47±0.17 (6)	6.74±0.42 (6)	6.24±0.21 (5)	5.25±0.47 (5)
AGD on PND1 (mm/g$^{1/3}$)	1.92±0.07 (5)	1.88±0.04 (6)	1.98±0.11 (6)	1.90±0.05 (5)	1.72±0.04 (5)
Testicular descent (day)	19.9±0.2 (5)	19.7±0.2 (6)	19.6±0.2 (6)	20.3±0.2 (5)	19.7±0.3 (5)
Female					
Body weight on PND1 (g)	5.69±0.12 (5)	6.25±0.19 (6)	6.19±0.35 (6)	5.89±0.20 (5)	5.09±0.47 (5)
AGD on PND1 (mm/g$^{1/3}$)	1.01±0.04 (5)	0.94±0.05 (6)	1.06±0.07 (6)	1.08±0.09 (5)	0.99±0.05 (5)
Vaginal opening (VO; day)	33.7±0.3 (25)	33.9±0.4 (30)	34.4±0.4 (31)	35.1±0.5 (25)[a]	27.8±0.4 (24)[b]
Body weight on VO (g)	114.2±2.7 (25)	118.4±2.5 (30)	122.4±2.5 (31)[a]	122.7±2.7 (25)[a]	65.0±2.5 (24)[b]

Effects of the exposure to three estrogenic compounds on the sexual development were analyzed using Fisher's PSLD test. All data are expressed as mean±S.E.M. (number).
[a] $P < 0.05$ vs. control group.
[b] $P < 0.001$ vs. control group.

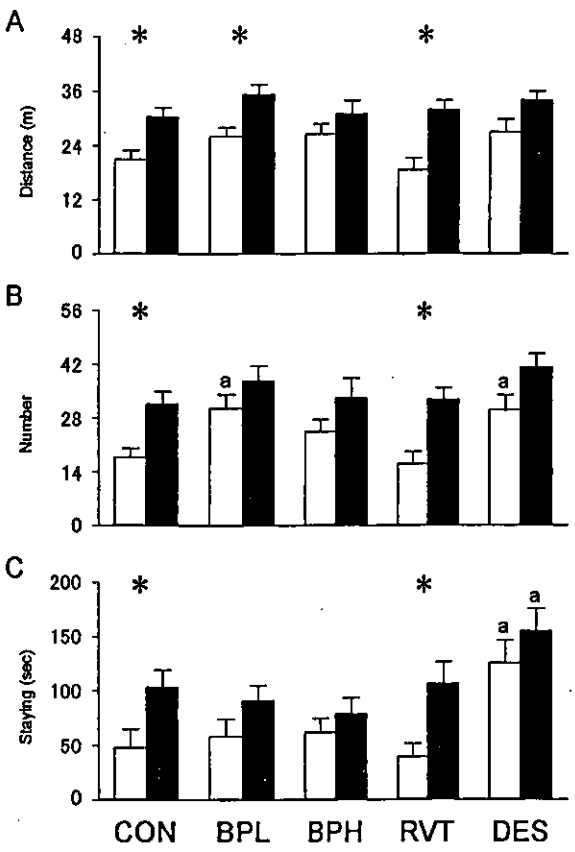

Fig. 1. The effects of estrogenic compounds on the open field behavior, including the total distance which rats moved (A), the frequency of rearing (B) and the duration of staying in the center area (C). The white and black columns are expressed as mean±S.E.M. for the male and female data, respectively ($n = 20$–24 animals per group). *, $P < 0.05$, Student's t-test between the male and female data. [a], $P < 0.05$ vs. control group in each sex.

in the control group (Fig. 2A). In addition, the number of intromissions did not change among any of the groups, while the intromission rate decreased in the BPL and RVT groups but not in the BPH or DES groups (Fig. 2B). On the other hand, the three estrogenic compounds had no influence on the duration from the first mount until the first ejaculation, the latency to first ejaculation, or the postejaculatory interval (Fig. 2C–E). From these data, we concluded that the male sexual behavior did not change remarkably due to exposure to either BPA, RVT or DES.

In the female animals, there was no difference in either the number of ear wiggles, the lordosis quotient, or the rejection score between the BPA-exposed group and the control group (Fig. 2F–H). In both RVT and DES groups, however, the lordosis quotient decreased while the rejection score increased, although the number of ear wiggles did not change. Since the lordosis quotient conflicts with the rejection in female sexual behavior, the reduced lordosis quotient corresponds with an increase in the rejection behavior, thus suggesting that both DES and RVT led to a reduced receptivity. As a result, BPA did not affect the female reproductive behavior while RVT and DES did.

3.4. Reproductive organs and serum hormone levels

The weights of both the male and female reproductive organs and the data of the sperm are shown in Table 2. BPA and RVT exposure induced no remarkable change in the weights of the epididymis, ventral prostate and seminal vesicle, although the weight of the testis increased only in the male offspring of the BPH group. In the male DES-treated rats, however, the weights of the testis and epididymis decreased while the weight of the seminal vesicle increased. None of the total spermatid in the left testis, the caudal sperm, or the percentage of motile sperm differed among the groups exposed to BPA, RVT or DES, thus suggesting that these compounds did not affect the quantity or the quality of the sperm. In the female offspring, three estrogenic chemicals induced no significant change in the weights of the

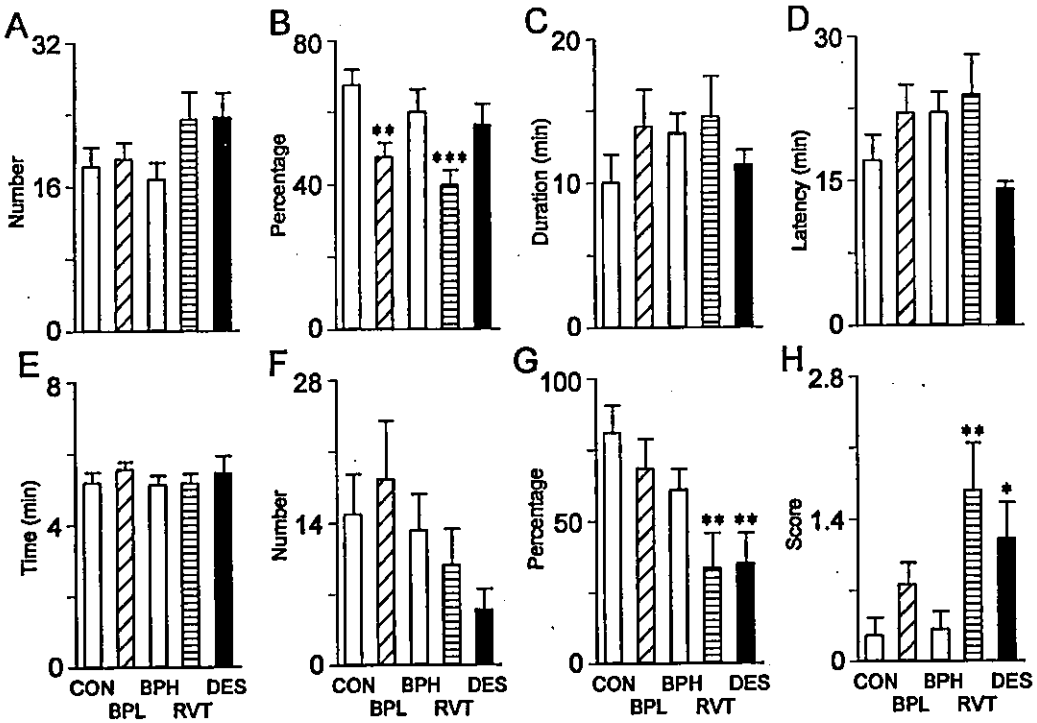

Fig. 2. The effect of three estrogenic compounds on the male and female sexual behavior. The number of mounts (A), the intromission rate (B), the duration from first mount to first ejaculation (C), the latency to first ejaculation (D), and postejaculatory interval (E) are expressed as mean ± S.E.M. ($n = 7–13$ animals per group) in males. The number of ear wiggles (F), the lordosis quotient (G), and the rejection score (H) are also expressed as mean ± S.E.M. ($n = 7–13$ animals per group) in females. The white, hatched, gray, striped and black columns show the control, BPL, BPH, RVT and DES groups, respectively. **, $P < 0.01$ vs. control group in each sex. ***, $P < 0.001$ vs. control group in each sex.

uterus or bilateral ovaries. The estrus cycles in the BPA-exposed groups were comparable to those in the control group, whereas the DES-treated females as well as the RVT-exposed females showed the abnormal estrus cycles, particular a reduced number of normal cycles. A histopathological study demonstrated that no significant change was observed either in the testes or in the ovaries.

Table 2
Effect on the reproductive organs

	Control	BPL	BPH	RVT	DES
Male	$n = 13$	$n = 15$	$n = 13$	$n = 12$	$n = 11$
Body weight at necropsy (g)	455.2 ± 4.1	460.8 ± 6.9	454.1 ± 8.2	458.8 ± 10.0	415.5 ± 7.6[d]
Testis (g)	1.821 ± 0.036	1.791 ± 0.037	1.981 ± 0.041[c]	1.862 ± 0.016	1.664 ± 0.028[c]
Epididymis (g)	0.556 ± 0.012	0.559 ± 0.012	0.566 ± 0.012	0.535 ± 0.008	0.517 ± 0.011[b]
Ventral prostate (g/100 g BW)	0.095 ± 0.003	0.085 ± 0.003	0.090 ± 0.004	0.085 ± 0.005	0.107 ± 0.004
Seminal vesicle (g/100 g BW)	0.127 ± 0.004	0.123 ± 0.004	0.134 ± 0.004	0.117 ± 0.003	0.141 ± 0.005[b]
Sperm in males	$n = 12$	$n = 15$	$n = 13$	$n = 11$	$n = 10$
Spermatid ($\times 10^6$ counts per testis)	128.5 ± 6.9	118.5 ± 4.6	136.0 ± 4.7	129.5 ± 5.0	143.8 ± 8.2
Sperm ($\times 10^6$ counts per cauda)	154.0 ± 10.2	161.1 ± 5.7	166.6 ± 6.7	161.1 ± 7.0	134.4 ± 6.4
Percent of motile sperm	80.3 ± 2.6	79.4 ± 2.1	80.9 ± 2.1	80.8 ± 1.7	78.2 ± 4.5
Female	$n = 13$	$n = 15$	$n = 15$	$n = 11$	$n = 14$
Body weight at necropsy (g)	274.9 ± 5.1	271.7 ± 5.0	270.4 ± 5.1	263.8 ± 6.6	262.3 ± 7.0
Uterus (g/100 g BW)	0.249 ± 0.013	0.245 ± 0.017	0.229 ± 0.010	0.229 ± 0.014	0.215 ± 0.011
Bilateral ovaries (g/100 g BW)	0.041 ± 0.002	0.042 ± 0.002	0.039 ± 0.001	0.041 ± 0.002	0.048 ± 0.004
Rats cycling normally[a]	86	72	78	19[d]	25[d]

Effects of the exposure to three estrogenic compounds on the reproductive organs were analyzed using Fisher's PSLD test. All data are expressed as mean ± S.E.M.

[a] Values are in percentage. A normal cycle is 4–5 days in length and consisted of four stages.

[b] $P < 0.05$ vs. control group.

[c] $P < 0.01$ vs. control group.

[d] $P < 0.001$ vs. control group.

Table 3
Effect on the serum hormone levels

	Control	BPL	BPH	RVT	DES
Male	$n=13$	$n=15$	$n=13$	$n=12$	$n=11$
LH (ng/ml)	3.55±0.21	3.66±0.27	3.55±0.22	3.56±0.19	3.37±0.28
FSH (ng/ml)	16.9±0.7	15.4±0.7	17.1±1.0	17.6±0.7	14.2±1.4
Prolactin (ng/ml)	36.8±6.1	42.3±8.2	50.6±14.2	22.6±3.5	36.7±5.3
Testosterone (ng/ml)	117.8±18.6	109.3±16.8	116.4±15.5	71.9±17.3	72.6±19.1
Female	$n=12$	$n=15$	$n=15$	$n=11$	$n=14$
LH (ng/ml)	4.00±0.23	4.95±0.36	4.38±0.38	3.96±0.28	4.70±0.51
FSH (ng/ml)	8.36±0.37	9.67±0.69	9.78±0.54	9.87±0.37	11.32±1.30
Prolactin (ng/ml)	39.1±8.0	54.7±8.0	36.2±12.5	54.7±12.3	26.9±4.0
17β-Estradiol (pg/ml)	18.7±1.8	19.5±2.1	13.9±1.4	15.8±1.7	12.9±1.9[a]

Effects of the exposure to three estrogenic compounds on the levels of serum hormones were analyzed using Fisher's PSLD test. All data are expressed as mean±S.E.M.
[a] $P<0.05$ vs. control group.

Table 3 shows the serum hormone levels of LH, FSH, prolactin and gonadal steroids (testosterone for males and 17β-estradiol for females). In both the male and female offspring, none of LH, FSH, or prolactin was influenced by any estrogenic chemical. Neither the testosterone nor 17β-estradiol levels changed significantly in the BPA-exposed group. In contrast, the levels of testosterone in the RVT and DES groups decreased, however the data were not statistically significant. The serum level of 17β-estradiol significantly decreased in the DES-treated group.

3.5. Sexually dimorphic nuclei

Three estrogenic compounds affected the sexually dimorphic nuclei in the brain in a different manner (Figs. 3 and 4). In all groups, the male brain was significantly heavier than the female brain and the volume of the male SDN-POA was several-times greater than that of the female SDN-POA. These results suggest that neither treatment affected the sex differences in the brain weight or the SDN-POA size (Fig. 3A) and they are also consistent with our previous findings (Kubo et al., 2001). On the other hand, the volume of the female LC was significantly larger than that of males in the control group, whereas the volume of the female LC was significantly smaller than that of males in the BPA- and DES-treated groups (Fig. 3B). In the RVT group, the sex difference of the LC size was not reversed but instead was abolished. Similar to the size of the LC, the sex difference regarding the cell number in the LC also reversed in the BPA- and DES-exposed groups while the cell number of the male LC was almost the same as that of females in the RVT group (Fig. 3C). These results suggest that the sensitivity to estrogenic chemicals is different between the LC and the SDN-POA.

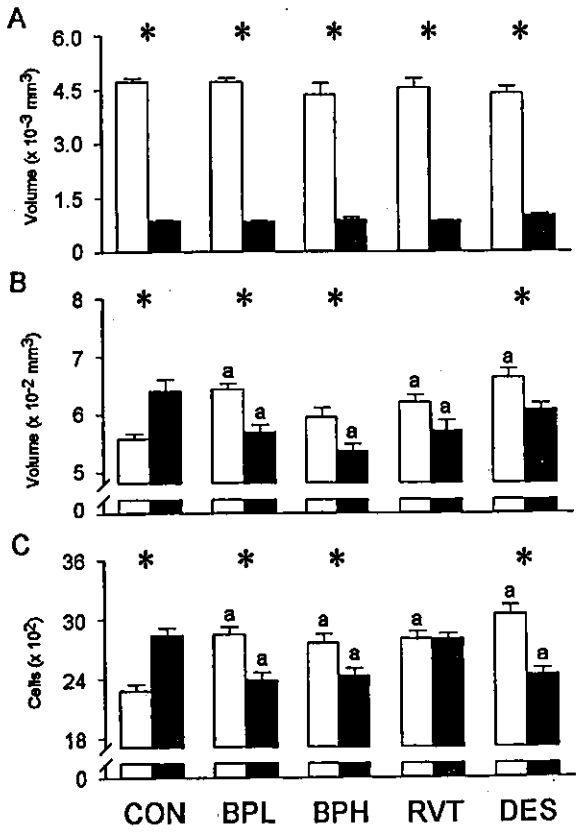

Fig. 3. The effects of three estrogenic compounds on the sex differences of the SDN-POA and the LC. The SDN-POA volume of males was greater than that of females in all groups (A). Though the female LC was larger than the male LC in the control group, the female LC was smaller than the male LC in the BPA and DES groups and there was no sex difference in the RVT group (B). The statistical significance of the number of neurons in the LC was similar to that of the LC size (C). The white and black columns are expressed as mean± S.E.M. for the male and female data, respectively ($n=7$–8 animals per group). *, $P<0.05$, Student's t-test between the male and female data. [a], $P<0.05$ vs. control group in each sex.

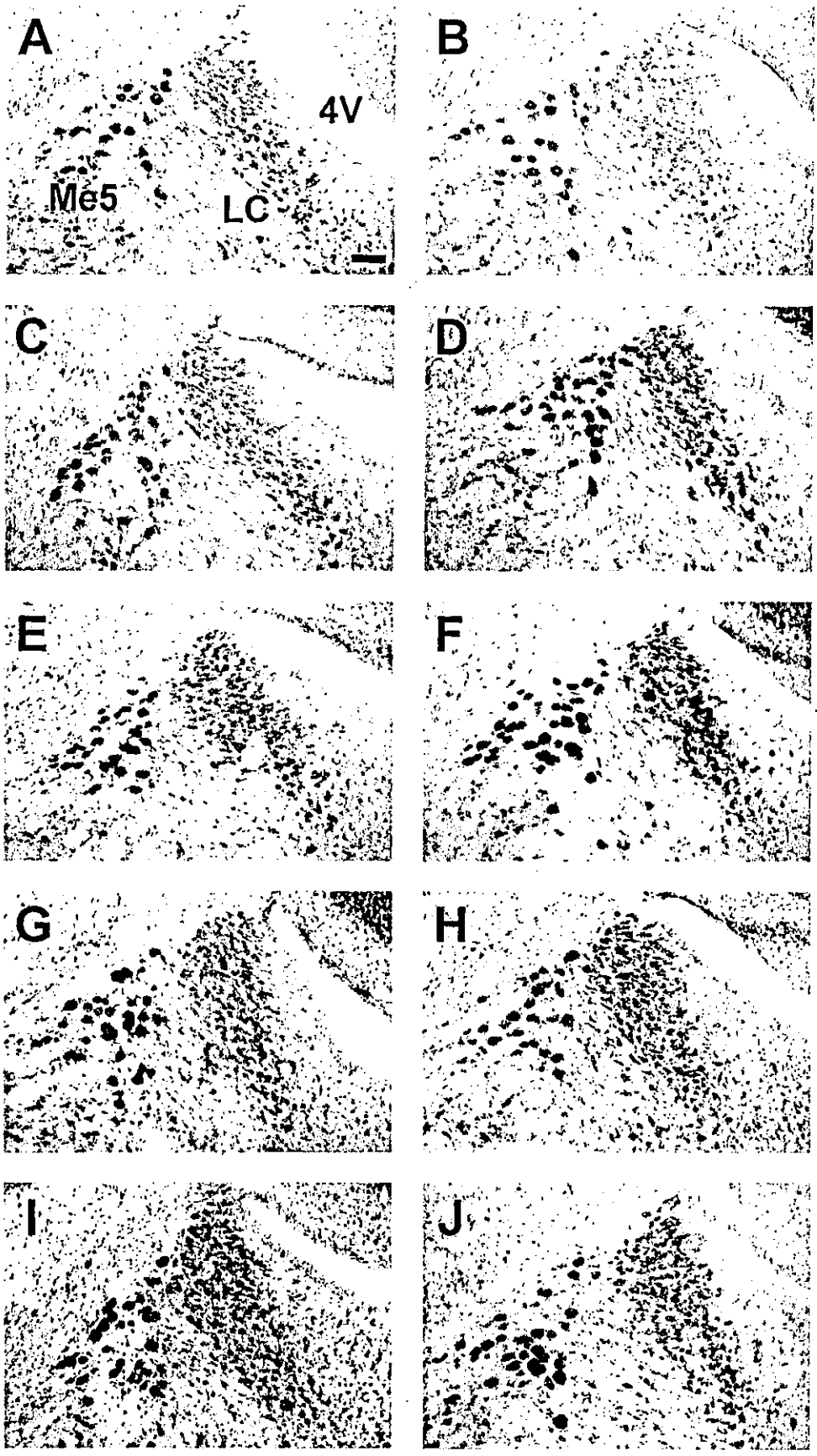

Fig. 4. Photomicrographs of the anterior intermediate region of the LC in males (A, C, E, G and I) and females (B, D, F, H and J). Frozen sections of the LC were stained with Cresyle violet in the control group (A and B), the BPL group (C and D), the BPH group (E and F), the RVT group (G and H) and the DES group (I and J). Me5, the mesencephalic trigeminal nucleus; 4V, the fourth ventricle. Bar, 0.1 mm. Magnification, ×200.

4. Discussion

The present study provides the first evidence indicating that the exposure of BPA during the fetal and suckling periods, even at a very low dosage below the TDI, disrupts the normal sexual differentiation in both the brain structure and behavior without any adverse effect on the reproductive system (see Table 4). We noted a disruption of sexual differentiation to be permanently initiated as an organizational effect, because these chemicals were exposed to the offspring only through placental translocation and milk from their mothers, and none of the offspring were exposed to any chemical substances after weaning. The human TDI are usually determined by multiplying the NOAEL and the safety index together, and any dosage under the TDI must not cause the adverse effect. Since BPA induced a permanent change in the CNS at the dosage below the TDI under the NOAEL in this study, our results suggest that it may be necessary to reassess the TDI of BPA, probably as well as the TDIs of other EEDs, using the endpoints regarding the sexual differentiation of the CNS, and that both the open-field behavior and the LC volume may become useful endpoints to determine the TDI.

The open-field behavior in the BPA-exposed male rats tended to be more similar to the typical behavior observed in control females than to that in the control males, even in the group exposed to BPA below the TDI. Maternal behavior is a factor which influences the open-field behavior (Caldji et al., 1998), but neither the nesting behavior nor the retrieval of pups in the treated groups differed from those of the control animals (unpublished observation). Therefore, the disrupted sex differences in this study were not caused by changes in the maternal behavior. It is well known that the perinatal exposure to estrogen plays a crucial role in the induction of sexual dimorphism in open-field behavior, based on the findings that the sex difference of open-field behavior is abolished in rats when they are exposed to testosterone or estradiol in the perinatal critical period (Blizard et al., 1975; Slob et al., 1981). In the present study, the effect of a potent estrogen, DES, was similar to that of BPA on open-field behavior. Therefore, the disrupted sex differences in the open field test may be due to, at least in part, an estrogenic effect of BPA. However, we cannot rule out the possibility that BPA acted as an antiestrogen, because open-field behavior in the BPA-treated rats was similar to that in the ERα-deficient mice (Ogawa et al., 1997). Further studies are needed to clarify the mechanism of the effect of BPA on open-field behaviors.

BPA exposure at any doses did not change the volume of the SDN-POA, and this finding is consistent with those of our previous study as well as other studies (Nagao et al., 1999; Kwon et al., 2000; Kubo et al., 2001). In contrast to the SDN-POA, we found that the exposure of BPA increased the male LC volume and decreased the female LC volume and as a result the sex difference in the LC structure was reversed. These results suggest that the LC may be more sensitive to BPA than the SDN-POA. The original phenotype of the brain is female type in mammals and the brain is only masculinized in males by 17β-estradiol converted from testosterone via aromatase in the neurons in the perinatal critical period (MacLusky and Naftolin, 1981; Lephart, 1996). Although females synthesize 17β-estradiol in their ovaries, the female brain is not modified during the same period due to the fact that 17β-estradiol is bound to α-fetoprotein and therefore

Table 4
Summary

		BPL	BPH	RVT	DES
Sex difference in the non-reproductive function					
Open field behavior	Locomotion	–	M = F	–	M = F
	Exploration	M = F	M = F	–	M = F
Locus coeruleus		M↔F	M↔F	M = F	M↔F
Male reproductive function					
Sexual development		–	–	–	–
Reproductive system	Organs	–	–	–	toxic
	Testosterone	–	–	–	–
Sexual behavior		M↓	–	M↓	–
Female reproductive function					
Sexual development		–	–	F↓	F↑↑
Reproductive system	Organs	–	–	–	–
	Estrus cycle	–	–	F↓	F↓
	17β-estradiol	–	–	–	F↓
Sexual behavior		–	–	F↓	F↓

–, No effect; M = F and M↔F, sex difference was abolished and inverted, respectively, M↓, demasculinization in males; F↓ and F↑↑, defeminization and feminization in females, respectively.

cannot pass through the blood brain barrier (Payne and Katzenellenbogen, 1979; Milligan et al., 1998). Evidence shows that the volume and number of neurons in the female LC decreased by the neonatal injection of testosterone and were comparable with those of the normal male LC (Guillamón et al., 1988). Therefore, the change of the LC in the present study may be due to some estrogenic action of BPA. ERα has been recently reported to prevent neurons from causing neuronal apoptosis while ERβ induces neuronal apoptosis (Nilsen et al., 2000). Both ERα and ERβ are expressed in the LC (Shughrue et al., 1997), but their expression patterns at various developmental stages are not known in the LC. Therefore, further study is needed to clarify the mechanism of the effect of BPA on the LC.

In the present study, we used DES as a representative positive control. As expected, both reproductive and non-reproductive systems were sensitive to DES (see Table 4), and these results are consistent with the findings of other studies (MacLusky and Naftolin, 1981; Csaba et al., 1986; Sharpe et al., 1995; Lephart, 1996; Ashby et al., 1997; Nagao et al., 1999; Kwon et al., 2000; Atanassova et al., 2000). In addition, we found that DES exposure increased the male LC volume and decreased the female LC size. These findings strongly support the hypothesis that the effect of BPA observed in this study originated from its estrogenic property. However, the female SDN-POA was not affected by the exposure of DES. It is well known that the perinatal or neonatal exposure of DES enlarged the size of the SDN-POA in female rats but not in males (Döhler et al., 1984; Tarttelin and Gorski, 1988; Vancutsem and Roessler, 1997; Kwon et al., 2000). A possible explanation for this discrepancy in females are that the dosage of DES used in this study are lower than in other studies (Döhler et al., 1984; Tarttelin and Gorski, 1988; Vancutsem and Roessler, 1997; Kwon et al., 2000). In their studies, the doses of DES were high enough to cause small and anovulatory ovaries in females, while the dose of DES used in the present study was lower and thus not sufficient to induce changes in the weight of the ovaries, AGD, or the size of the SDN-POA. This hypothesis is supported by the finding showing that the females with longer AGDs at PND 1 had significantly larger SDN-POA volumes than those with short AGDs in the neonatally DES-treated rats (Faber et al., 1993).

We tested the effect of RVT as another type of estrogenic control in this study and we found novel evidence that the exposure to RVT during the fetal and suckling periods mainly affected the reproductive function (see Table 4). The inhibited sexual behavior in the RVT-treated females probably resulted from the estrogenic disruption of the female copulatory behavior, because the perinatal exposure of estrogens including DES has been shown to inhibit the female sexual behavior in adulthood in previous studies (MacLusky and Naftolin, 1981; Csaba et al., 1986; Lephart, 1996; Nagao et al., 1999). RVT abolished but did not invert the sex difference of the LC volume, in contrast to BPA and DES. Since the common characteristic of BPA, DES and RVT is to have an estrogenic action, the changes in the LC volume caused by these three compounds may be due to their estrogenic action. The estrogenicity of RVT seems to be less than those of BPA and DES, at least for the LC ontogeny. In this study, a vaginal opening delayed in the RVT-treated females. The perinatal exposure to estrogen accelerates vaginal opening and therefore RVT-induced delay of vaginal opening seems to contradict the estrogenic property of RVT. However, the perinatal exposure to androgens, aromatase inhibitor (fadrozole) and genistein is known to cause a delay in vaginal opening (Levy et al., 1995; Marty et al., 1999). According to the fact that RVT as well as genistein can act as a mixed agonist/antagonist rather than an agonist for the estrogen receptors (Miodini et al., 1999; Bowers et al., 2000), RVT may antagonize the estrogenic acceleration of vaginal opening.

To our knowledge, the effects of very low-doses of industrial artificial chemicals on the CNS have rarely been investigated. An important question remains regarding whether the disruption of sexual differentiation in the CNS are caused in humans or not. Based on our limited data, we do not know whether human exposure to BPA during the fetal, neonatal, and infantile periods result in any adverse effects on the human CNS, because the level of human exposure is likely to be lower than that used in our treatments. However, it is likely that the summation of estrogenic and/or other effects of each endocrine disrupter impairs the sexual differentiation of the brain and behavior, because we are exposed to a number of artificial chemical substances, in which many compounds are estrogenic such as BPA.

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Advances in Brief

Etiology-specific Gene Expression Profiles in Rat Mammary Carcinomas[1]

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Abstract

Identification of etiology of human cancers is important for effective cancer prevention, and attempts to estimate the roles of a variety of environmental carcinogens in human cancers are being made. Here, we applied cDNA microarray technology to estimate whether gene expression profiles of cancers would reflect their etiology. Using rat mammary carcinoma models, expression profiles were analyzed in two groups of carcinomas induced by distinct carcinogens but with the same histological classification. Four carcinomas induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) and three carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and a high-fat diet were analyzed by a GeneChip oligonucleotide microarray that contained ~8000 rat genes. By hierarchical clustering analysis, the seven carcinomas were classified into two groups that exactly coincided with the DMBA-induced and the PhIP-induced groups. The correlation coefficient between the two groups was 0.63, and those between any carcinomas within each group ranged from 0.78 to 0.95. In addition, characteristic clusters of genes were also identified that highlighted distinct and common characteristics of both groups. Seventeen genes were down-regulated in the DMBA and up-regulated in the PhIP-induced groups. Thirty-three genes were regulated in the opposite manner. Our results indicated that gene expression profiles in cancers reflect their etiology and suggested a possibility that etiology of cancers could be retrospectively estimated from their expression profiles.

Introduction

Clarification of the etiology of human cancers and estimation of their roles are some of the most important issues in cancer research. If specific contributions of environmental carcinogenic factors to human cancers could be estimated, it would be possible to take reasonable procedures to avoid them with minimal expense and sacrifice of daily comfort. Epidemiology has played an important role in identification of major carcinogenic factors, such as smoking, viral infection, and carcinogens in diet, and the estimation of their roles in human cancers. However, there are many more carcinogenic factors in the environment, the roles of which in carcinogenesis are difficult to be estimated only with traditional epidemiology (1–3). Molecular epidemiology is one of the approaches to estimate the carcinogens involved in human cancers, making use of signature mutations left in the cancer (4, 5).

cDNA microarray technology has provided new information in many aspects of tumor biology (6). The expression profiles in cancers were shown to reflect their histological characteristics, clinical outcomes, and the responses to treatment (7–12). In addition, the expression profiles were used to develop clinical biomarkers (13–15). However, no reports are available regarding the correlation between the etiology of human cancers and their gene expression profiles, except for hepatocellular carcinomas caused by hepatitis viruses (16). This is because the etiologic factors of human cancers are complex and very difficult to determine.

For this reason, animal models are expected to provide good resources, as animal cancers are induced by defined protocols and the carcinogenic factors involved are clear. Rat mammary carcinomas can be reproducibly induced by well-established methods using DMBA[3] (17), PhIP (18), and NMU (19). The induced mammary carcinomas predominantly originate from mammary ducts (17) and show smaller phenotypic variations (20) and fewer genetic alterations than the human counterpart (21, 22). In addition, normal mammary glands can be purely isolated from inguinal fat by the gland isolation technique.

In this study, we analyzed expression profiles of rat mammary carcinomas induced by DMBA and those induced by PhIP, a representative food-born carcinogen (23), using a cDNA microarray, and we classified these carcinomas based on their gene expression profiles and estimated whether the profiles reflect their etiology.

Materials and Methods

Mammary Carcinomas and Normal Mammary Ducts. To induce mammary carcinomas by DMBA, 30 female (F344 × SD)F_1 rats at the age of 7 weeks were administrated a single dose of DMBA (50 mg/kg) in corn oil by gavage. Mammary carcinomas were induced in 16 of 30 rats at the ages of 25–32 weeks. To induce mammary carcinomas by PhIP and high-fat diet, 33 female (F344 × SD)F_1 rats were given 10 doses of PhIP (75 mg/kg/day) in water at 6 weeks of age by gavage and were fed a diet with 23.5% corn oil. Mammary carcinomas were induced in 15 of 33 rats at the ages of 56–72 weeks (24). Macroscopic tumors were histologically examined by two experienced pathologists (K. M., S. F.). Normal mammary ducts were collected from age-matched untreated female (F344 × SD)F_1 rats by the gland isolation technique for mammary ducts (24). The tumors and mammary ducts were kept frozen at −80°C until extraction of total RNA. Total RNA was isolated by Isogen (Nippon Gene, Tokyo, Japan).

GeneChip Protocol. GeneChip Rat Genome U34A arrays, which contained probe sets for ~8000 rat genes, were purchased from Affymetrix (Santa Clara, CA). Five μg of total RNA were used as starting material for cDNA preparation. The first-strand cDNA was synthesized with the SuperScript II reverse transcriptase (Invitrogen, Groningen, The Netherlands) and a T7-$(dT)_{24}$ primer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The double-strand cDNA was synthesized with *Escherichia coli* RNase H, *E. coli* DNA polymerase I, and *E. coli* DNA ligase (Toyobo, Tokyo, Japan). Biotin-labeled cRNA was prepared using a HighYield RNA transcript labeling kit (Affymetrix). After *in vitro* transcription, the unincorporated nucleotides were removed using the RNeasy Mini kit (Qiagen, Valencia, CA). Twenty μg of each labeled cRNA were fragmented, and its quality was assessed by gel electrophoresis.

The labeled cRNA was placed in a hybridization mixture containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre), as recom-

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; NMU, *N*-methyl-*N*-nitrosourea; RT-PCR, reverse transcription-PCR.