Western blot analyses; Cells were washed with phosphate-buffered saline and directly lysed with 2 x SDS-sample buffer. The lysates were sonicated with a ultrasonic devise, heated and separated in polyacrylamide gels. The proteins were transferred to PVDF membranes (GE healthcare, Tokyo, Japan). Bcl-2 or methylated histone H3 was detected using an anti–Bcl-2 antibody (50 ng/ml, Santa Cruz Biotechnology, Santa Cruz, CA) or anti–monomethyl histone H3 (lys9) antibody (MAB Institute, Sapporo, Japan), respectively. Bound antibodies were visualized with a peroxidase-labeled, anti–mouse IgG antibody (100 ng/ml, Jackson ImmunoResearch Laboratories, West Groves, PA) and Chemilumi super (Nacalai Tesque, Kyoto, Japan). The same PVDF membranes, from which bound antibodies were stripped, were reprobed with an anti–α-tubulin DM1A antibody (50 ng/ml, BioCarta, San Diego, CA) to detect α-tubulin. For detection of FLAG-GPR30, cells were homogenized in TED buffer (20 mM Tris-Cl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.5) containing 1 x protease inhibitor cocktail (Nacalai Tesque). The homogenates were centrifuged at 800 x g for 5 min, and the supernatants were further centrifuged at 20,000 x g for 20 min. The resultant cell membranes (50 μg) were subjected to western bloting using an anti–DYK antibody (100 ng/ml, Wako Pure Chemicals).

Densitometric analysis; For densitometric analysis, the chemiluminescent signal was detected by exposing the membranes to x-ray films (RX-U, Fuji film, Tokyo, Japan) for 15 to 180 sec, and the film was developed. Exposure times that did not saturate the signal in the darkest band on each gel were used for the quantitative analysis of signals. The developed film was digitized using a flat-bet scanner (GT9400UF, Epson, Japan)

and densitometric and quantitative analysis of signals was performed using ImageJ (NIH).

[^{35}S]-GTPγS binding assay; [^{35}S]-GTPγS binding assay was performed as described previously [18]. Cell membranes (50 μg) were incubated with 0.1 nM [^{35}S]-GTPγS (0.1 μCi per assay, American Radiolabeled Chemicals, St. Louis, MO), 10 μM GDP, and drugs at 32 °C for 30 min in binding buffer (20 mM Hepes-Na, 100 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, pH 7.5) containing 0.05% fatty acid-free bovine serum albumin (Nacalai Tesque). The bound [^{35}S]-GTPγS was separated from free by rapid filtration through GF/C filters, and the filters were counted by liquid scintillation.

Statistical Analysis; Analysis of variance (ANOVA) followed by a post hoc test was applied to data to determine statistical significance by using the statistical software, StatView 4.5 (Abacus Concepts, Berkeley, CA).

Results

Expression of Gpr30 gene in Swiss 3T3 fibroblast cells

RT-PCR analyses revealed that Swiss 3T3 fibroblast cells expressed *Gpr30* gene but not *Esr1*, *Esr2*, nor *Esrrγ* genes (Fig. 1). Although 3T3 fibroblasts also showed expression of *Esrrα* and *Esrrβ* genes, their endogenous ligands have been yet unidentified and BPA has been unknown to bind to ERRα or ERRβ. Thus, the use of 3T3 fibroblasts would provide the hint for understanding the mechanisms mediated through GPR30 or the non-estrogenic pathway.

Effects of BPA or NP exposure on H_2O_2-induced cell death and bcl-2 expression in 3T3 fibroblasts

We examined whether BPA or NP treatment of 3T3 fibroblasts affected the susceptibility against oxidative stress. When control 3T3 fibroblasts were serum-deprived and treated with H_2O_2 to cause oxidative injury, cell death was observed in a concentration-dependent manner with an EC_{50} of approximately 30 μM (Fig. 2A). 3T3 fibroblasts were exposed to varying concentrations of BPA for 30 ~ 45 days, then subjected to cell death assays. Cells treated with 1 nM BPA were resistant to 30 μM H_2O_2-induced cell death (Fig. 2B). Similar increase of resistance was observed when cells were exposed to NP for the same period (Fig. 2C). Therefore, long-term exposure of 3T3 fibroblasts to BPA or NP might influence cellular machineries sensitive to oxidative injury.

Next, we examined the expression of bcl-2, an anti-apoptotic protein, because increased expression of bcl-2 was reported to protect 3T3 fibroblasts from H_2O_2-induced cell death [19]. Thirty to forty days exposure of cells to BPA or NP apparently

increased the expression of bcl-2 protein in 3T3 fibroblasts (Fig. 2D). BPA was known to affect gene expression via epigenetic pathways [20,21], and tegulation for bcl-2 expression via histone methylation was also reported in breast cancer cells [22]. Therefore, we examined whether histone methylation was altered by exposure to BPA or NP in 3T3 fibroblasts. One to two weeks exposure resulted in decrease of histone H3 methyaltion (Fig. 2E).

By contrast to the enhanced cell survival, cell proliferation remained unchanged by BPA or NP exposure (Fig. 2F). No oncogenic characteristic, such as colony formation, piling up of cells, or destruction of contact inhibition, was also observed in cells exposed to chemicals for over one month (Fig. 2G).

We further performed the same cell death assay using 3T3 fibroblasts cultured with or without BPA or NP in D-MEM containing phenol red and FCS. Similar to the results shown in figure 2, BPA exposure for one week or one month, but not one day, caused resistance to H_2O_2 treatment in a concentration-dependent or inverted 'U' shape manner within a range of 0.1 ~ 100 nM (Fig. 3A~C). One week exposure of 3T3 fibroblasts to NP also resulted in increase of cell viability against H_2O_2 treatment in an inverted 'U' shape manner (Fig. 3D). This finding suggests that phenol red or other small molecules including steroid hormones present in FCS show no marked influence on BPA- or NP-induced effects under our culture conditions.

Effects of BPA on GPR30 activation

Given that Swiss 3T3 fibroblasts expressed only *Gpr30* gene, as shown in Figure 1, encoded GPR30 receptor would be a strong candidate for mediating the effect of BPA or NP. Previously, both compounds were reported to activate GPR30 using a

homologous expression system where the employed HEK293 cells endogenously expressed *Gpr30* (our unpublished data). Thus, we developed a heterologous expression system to examine whether BPA or NP could activate GPR30. For this purpose, we searched several cell types which showed no endogenous *Gpr30* gene expression, and found that a subline of RH7777 cells expressed no *Gpr30* gene. Then, we exogenously expressed FLAG-tagged GPR30 in RH7777 cells, and isolated a clonal cell line expressing GPR30, RH7777-GPR30 (Fig. 4A). For detection of GPR30 activation, [^{35}S]-GTPγS binding assay was employed, which has been established as a sensitive method detecting activation of $G_{i/o}$ or G_s-coupled G protein-coupled receptors, including GPR30 [18,23]. When cell membranes of RH7777-GPR30 cells were stimulated by varying concentrations of BPA or NP, concentration-dependent G protein activation was observed (Fig. 4B). By contrast, there was no increase of G protein activation in cell membranes from control RH7777 cells (Fig. 4B).

Discussion

In the present study, we found that long-term exposure of fibroblasts to BPA at lower concentrations resulted in the acquisition of resistance against oxidative injury. Similar effects were observed in fibroblasts exposed to another chemical, NP. These effects of BPA or NP were somehow in an inverted 'U' shape manner within a range of 0.1 to 100 nM. Mnay endocrine disrupters have been reported to show 'U' or inverted 'U' shape responses within a wide range of doses *in vivo* as well as *in vitro* [24,25]. The reasons why these non-monometric curves are observed in many experiments remain to be clarified. This may be due to the existence of two or more distinct targets for these chemicals. Alternatively, cells may intrinsically respond to maintain homeostasis when exposed to these chemicals at higher concentrations.

In addition to these non-monometric responses, in this study, the effects of BPA or NP were observed when fibroblasts were exposed to these chemicals at least one week, but not one day. Furthermore, in cell death assay, H_2O_2 was treated to fibroblasts under conditions where no chemical was exposed. These indicate that alterations of cell survival might need genomic changes during long-term exposure of fibroblasts to chemicals and that chemicals show no direct effect on H_2O_2-induced cell death. Indeed, we found that both BPA and NP could affect histone methylation, one of important epigenetic mechanisms, in 3T3 fibroblasts. Whether this epigenetic alteration directly controls bcl-2 expression would be an interesting issue to be explored.

BPA has been shown to induce various effects on many cell types *in vitro* and *in vivo*, including neuronal cells, immune cells, tumor cells, and endocrine cells [1,2]. Most, if not all, effects have been considered to be mediated via estrogenic activity through binding to nuclear or membrane receptors, including ERα, ERβ, ERRγ, or

GPR30. Swiss 3T3 fibroblasts used in this study expressed only *Gpr30* gene. Thus, GPR30 is likely to be a strong candidate responsible for the increased resistance to oxidative injury in 3T3 cells exposed to BPA or NP. Indeed, BPA and NP could activate GPR30 at the concentrations which showed increased cell viability.

A several lines of evidence indicate that GPR30 couples to G_s to increase cAMP and, indeed, BPA treatment with GPR30-expressing cells results in cAMP accumulation [6,23]. The cAMP-dependent pathway-mediated cytoprotective effects have been demonstrated to likely in part involve bcl-2 expression, because cAMP responsive element (CRE) exists in the promotor region of bcl-2 gene and GPR30 activation induces bcl-2 expression [26]. Bcl-2 inhibits cell death induced by oxidative stress in many cell types, including fibroblasts [19]. Taken together, in 3T3 fibroblasts, activation of the GPR30 downstream CRE-binding protein pathway that induces bcl-2 expression might be involved in BPA- or NP-mediated increase in resistance to oxidative injury. Because BPA has been shown to influence the expression of many other anti-apoptotic or proapoptotic molecules, such as bax or caspase [27], it would be possible that these molecules are also involved in the effects of BPA or NO observed in 3T3 fibroblasts.

However, an IC_{50} for BPA to GPR30 was reported to be 630 nM [28]. By contrast, the concentrations which show cell protection and GPR30 activation were as low as 1 nM in this study. This discrepancy remains unclear. It would be speculated that long-term exposure of cells to BPA or NP at lower concentrations may result in intracellular accumulation of chemicals, then effective stimulation of G protein activity through GPR30 present in endoplasmic reticulum. Nonetheless, intracellular localization of GPR30 is still under debate, and biological significance of GPR30 in

endoplasmic reticulum remains to be determined [29,30].

Alternatively, other non-receptor mechanisms may exist, such as direct DNA insults that could cause impairment of transcription or cell cycle, leading to altered cellular responses [31]. Although we observed no changes in cell proliferation, cellular senescence involving reduced cellular responses to environmental stimuli might occur. More detail analyses will be necessary.

Here we focus on the ability of fibroblasts to resist to oxidative injury. It is unclear whether this effect on cell survival is common or limited to certain cell types. However, given that estrogen could protect neuronal cells from oxidative injury, such protective effects of BPA or NP may be commonly observed in many cell types. Therefore, the present study provide with some ideas to understand molecular mechanisms through which BPA or NP influences cellular functions.

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Figure legends

Figure 1 Swiss 3T3 fibroblasts express *Gpr30* gene. Total RNAs were extracted from cells, or mouse hippocampus (Hip) or cerebral cortex (Cor), and subjected to RT-PCR to detect genes of estrogen receptors (*Esr1* and *Esr2*), *Gpr30*, estrogen related receptors (*Esrr*α, *Esrr*β, *Esrr*γ).

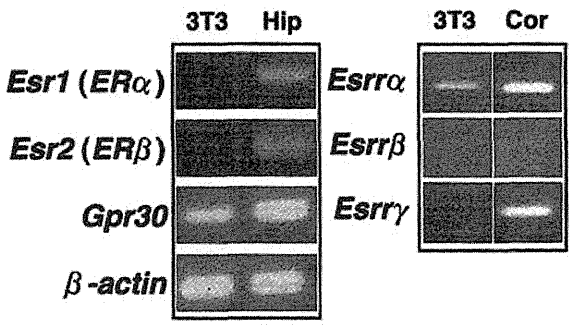
Figure 2 Long-term exposure to bisphenol A or nonylpnehol ameliorates H_2O_2-induced cell death in 3T3 fibroblasts. (A) Effects of H_2O_2 treatment on 3T3 cell survival. Fibroblasts were treated with varying concentrations of H_2O_2 for two days and the number of cells were determined. Data express % of control (no H_2O_2) and are the mean ± S.D. of quadruplicates determinations. (B) Effects of 30 ~ 45 days exposure to varying concentrations of bisphenol A on H_2O_2-induced cell death. Fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean ± S.E.M. of five independent experiments. *; $p < 0.05$ vs no H_2O_2. (C) Effects of 30 ~ 45 days exposure to varying concentrations of nonylphenol on H_2O_2-induced cell death. Fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean ± S.E.M. of five independent experiments. *; $p < 0.05$ vs no H_2O_2. (D) Effects of bisphenol A or nonylpnehol on bcl-2 expression in 3T3 fibroblasts. 3T3 fibroblasts were exposed to bisphenol A (BPA) or nonylphenol (NP) for 30 to 40 days, and subjected to western blot assay for bcl-2 or α-tubulin. The ration of bcl-2 amounts to α-tubulin amounts was densitometrically determined. Data express % of control, and are the mean ± S.E.M. of five independent experiments. *;$p<0.05$ vs DMSO. (E) Effects of bisphenol A or nonylpnehol on histone H3 methylation in 3T3 fibroblasts. 3T3

fibroblasts were exposed to bisphenol A (BPA) or nonylphenol (NP) for 7 to 14 days, and subjected to western blot assay for methylated histone H3 (H3K9Me) or α-tubulin. The ratio of H3K9Me amounts to a-tubulin amounts was densitometrically determined. Data express % of control, and are the mean ± S.E.M. of three independent experiments. *; $p<0.05$ vs DMSO. (F) Effects of bisphenol A or nonylpnehol on 3T3 fibroblast cell growth. 3T3 fibroblasts were exposed to bisphenol A (BPA) or nonylphenol (NP) for 45 days, and subjected to cell growth assay. Data express as fold increase of the absorbance to values at day 0 and are the mean ± S.D. of quadruplicates determinations from one representative experiment. (G) Phase contrast micrographs of 3T3 fibroblasts. Cells were maintained in the presence or absence of DMSO, BPA or NO for 30 days. Upper panel; cells were sparsely plated, and cultured for 1 day. Lower panels; cells are cultured for 2 days after they reached confluency. Bar indicates 78 μm for in upper panels, 250 μm in lower panels.

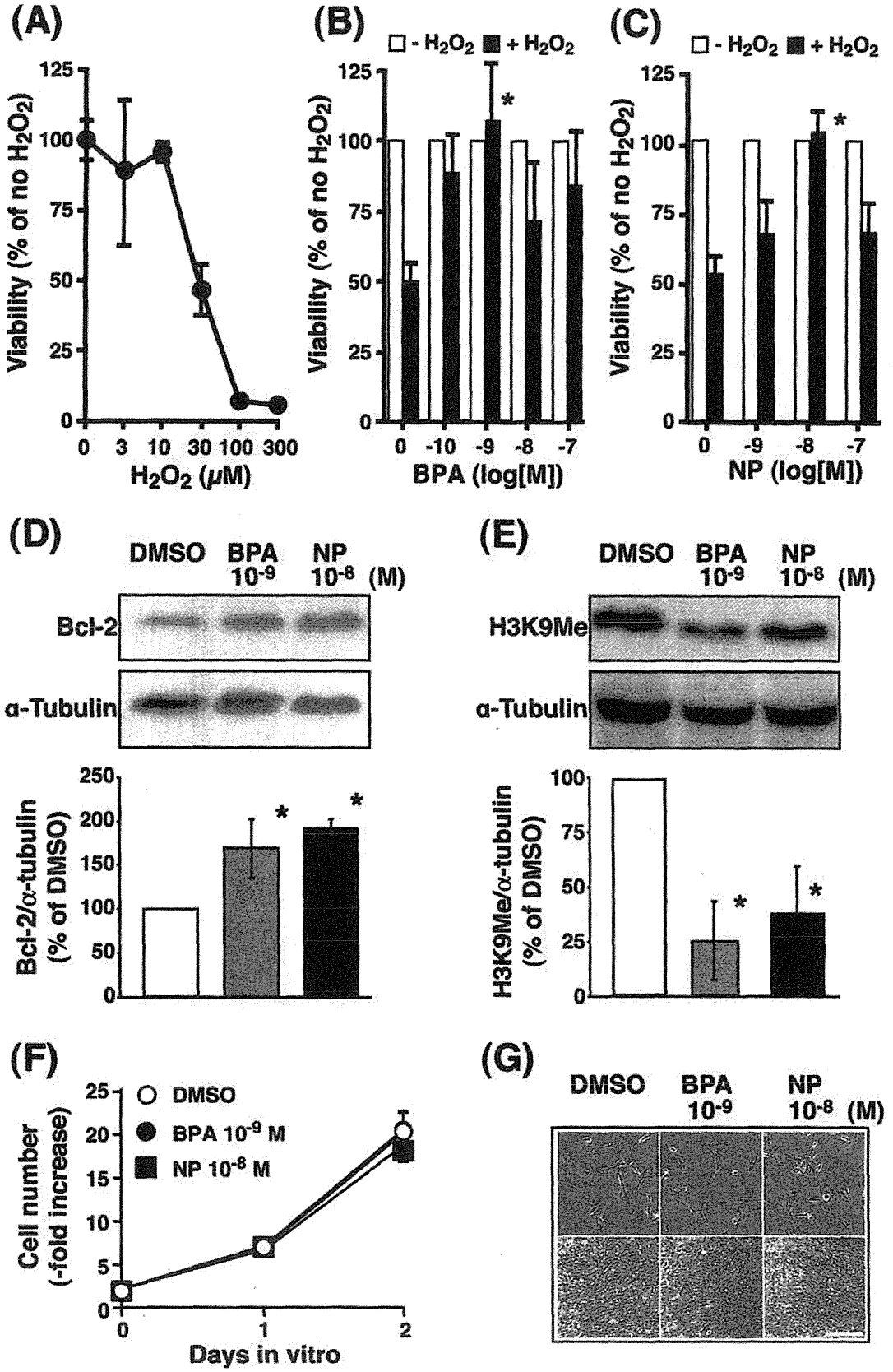
Figure 3 Bisphenol A or nonylpnehol exposure ameliorates H_2O_2-induced cell death in 3T3 fibroblasts cultured in D-MEM containing phenol red and fetal calf serum. (A) Effects of one month exposure to varying concentrations of bisphenol A (BPA) on H_2O_2-induced cell death. Fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean ± S.E.M. from more than three independent experiments. **; $p < 0.01$, *; $p < 0.05$ vs no H_2O_2. (B) Effects of one week exposure to varying concentrations of BPA on H_2O_2-induced cell death. Fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean ± S.E.M. from more than three independent experiments. *; $p < 0.05$ vs no H_2O_2. (C) Effects of one day exposure to

BPA on H_2O_2-induced cell death. Fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean ± S.E.M. of more than three independent experiments. (D) Effects of one week exposure to varying concentrations of nonylphenol (NP) on H_2O_2-induced cell death. 3T3 fibroblasts were treated with 30 μM H_2O_2 for two days. Data express % of no H_2O_2 treatment in each group, and are the mean ± S.E.M. of more than three independent experiments. *; $p < 0.05$ vs no H_2O_2.

Figure 4 Bisphenol A and nonylphenol induce GPR30-dependent G protein activation. (A) Establishment of RH7777-GPR30 cell line. Total RNAs or cell membranes were prepared from control RH7777 or RH7777-GPR30 cells and subjected to RT-PCR or western blot assay using anti-FLAG antibody, respectively. (B) [^{35}S]-GTPγS binding assay. Cell membranes were prepared from control RH7777 or RH7777-GPR30 cells and incubated with [^{35}S]-GTPγS in the absence or presence of varying concentrations of bisphenol A (BPA) or nonylphenol (NP). The bound [^{35}S]-GTPγS was separated and counted. Data express % of control (no drug) and are the mean ± S.E.M. from four independent experiments. *; $p < 0.05$ vs control.



[Fig. 1 Nishimura et al.]



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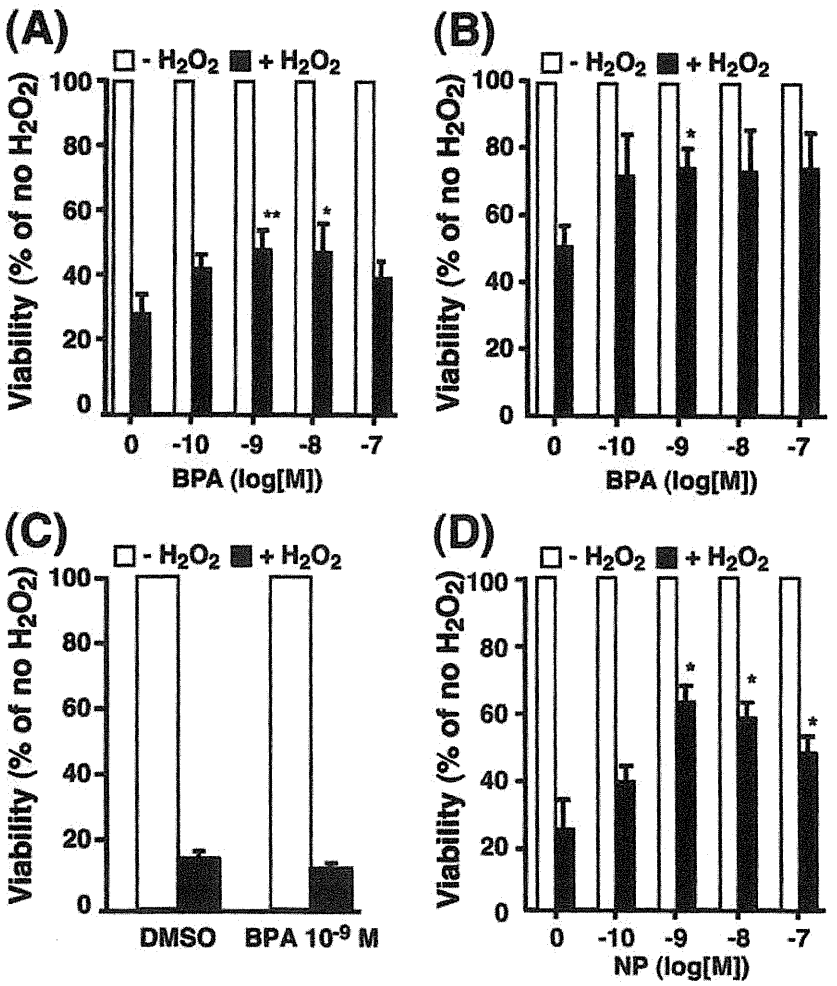

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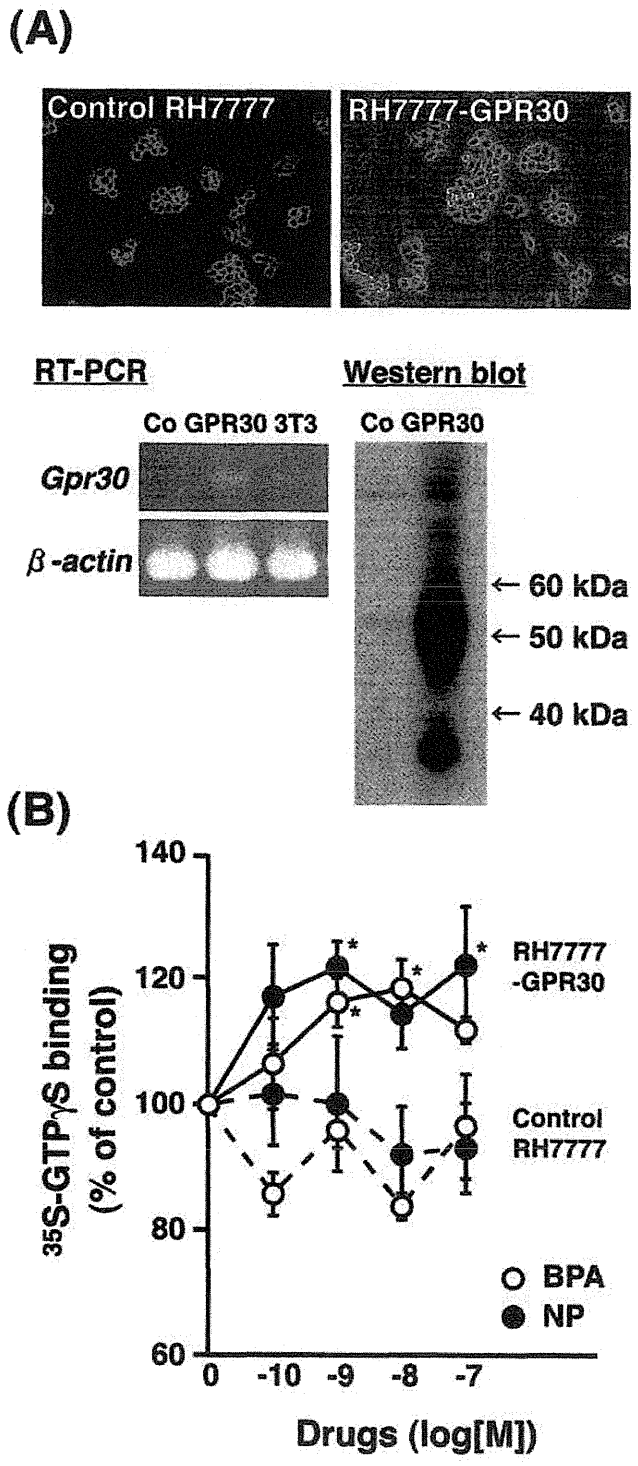

[Fig. 4 Nishimura et al.]

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SHORT COMMUNICATION

Early to middle gestational exposure to diethylstilbestrol impairs the development of labyrinth zone in mouse placenta

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ABSTRACT This study was performed to clarify the involvement of impaired labyrinth zone (LZ) of the placenta in the developmental toxicity of diethylstilbestrol (DES). DES at 10 μg/kg per day was administered orally to mice on days 4 through 8 of gestation. Histological observation of the LZ and determination of blood glucose levels in dam and fetus were performed on day 13. A high frequency of embryonic death was observed in the DES group. DES induced the underdevelopment of the plexus vasculosus, extensive maternal blood space and the decreased expression of glucose transporters in the LZ, and a reduction of the glucose level in embryos. These findings suggest that impaired LZ development may be related to the embryolethality of DES.

Key Words: development, diethylstilbestrol, embryotoxicity, labyrinth zone, placenta

INTRODUCTION

Placental growth and development are crucial for the development of the fetus, and dysfunction of the placenta may be closely related to fetal developmental disabilities (Bauer et al. 1998; Katayama et al. 2002; Cross 2006). The placenta is a highly vascularized tissue that develops during early gestation to facilitate the circulation of blood, oxygen, glucose and nutrients between the mother and the fetus. The rodent placenta comprises two morphologically distinct zones: the junctional zone (JZ) and the labyrinth zone (LZ) (Davies and Glasser 1968; Furukawa et al. 2011). The LZ of the placenta is the principal site of maternofetal exchange of substances. Namely, the LZ plays a role in transporting nutrients. Glucose is one of the most important substances transferred from the maternal blood to the fetal circulation in the placenta, and is a primary energy source for the fetus.

Diethylstilbestrol (DES) is a synthetic (stilbene) estrogen with an *in vivo* estrogenic potency similar to that of 17β-estradiol (E_2), and is structurally related to E_2. DES was widely used to prevent threatened miscarriages from the 1940s to 1971. In our previous study, we demonstrated that decidual hypoplasia and subsequent placental hemorrhage cause a high frequency of fetal death as a result of the oral administration of DES during the early stage of pregnancy in mice (Nagao and Yoshimura 2009). It has been shown that the incidences of embryonic mortality after treatment with DES at 0, 1, 5, 10 and 15 μg/kg per day were 2.8%, 5.4%, 5.5%, 68.5% and 88.2%, respectively, suggesting that the no observed adverse effect dose level (NOAEL) for embryonic lethality was 5 μg/kg per day upon oral exposure on days 4 through 8 of gestation and evaluation on day 13 in ICR mice (Nagao et al. 2012). In addition, we reported that DES affects the rough-surfaced endoplasmic reticulum of trophoblast giant cells and the developing LZ, and suggested that these developmental changes may be related to decreased protein synthesis or disruption of nutrition and oxygen exchange between mother and embryo (Nagao et al. 2013).

Thus, to address the adverse effects of oral DES administration on placentogenesis resulting in embryonic death or intrauterine growth restriction in mice, the present study was designed to examine the effects of DES administration on the development of the LZ, where most nutrient exchange between the mother and the embryo occurs.

MATERIALS AND METHODS

Animals and exposure to DES

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Kinki University (Permit Number: 27–2956). ICR mice at 8 weeks old purchased from CLEA (Osaka, Japan) were used. The mice were kept under SPF conditions and housed in polycarbonate cages in a room in which the temperature (23 ± 1°C), humidity (50 ± 5%) and light cycle (12:12 h light : dark cycle; lights on at 07.00 hours) were maintained. The mice were allowed food (CE-2, CLEA, Osaka, Japan) and drinking water *ad libitum*. Ten-week-old virgin female mice were cohabited for 2 h (07.00–09.00 hours) with males aged 11 weeks or older. The females were checked for the presence of a vaginal plug immediately thereafter, and the presence of a plug represented day 0 of gestation.

Diethylstilbestrol (DES, 3,4-bis[p-hydroxyphenyl]-2,4-hexadiene) was purchased from Sigma Chemical (St. Louis, MO, USA), suspended in corn oil and administered orally on days 4 through 8 of gestation, a time in the early to middle stage of placentogenesis (Tremblay et al. 2001). Ten mice administered corn oil were used as controls. Ten mice were administered DES at 10 μg/kg per day to address the developmental effects of DES on embryo viability. Administration occurred at a defined time (12.00 hours). On day 13 of gestation, seven pregnant mice in each group were euthanized using sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) anesthesia, and the uteri from dams in each group were removed to count the numbers of live and dead embryos as well as to measure live embryonic and placental weights, and the

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determination of sex was carried out by observation of the gonadal morphology and position under a dissecting microscope. The live and dead embryos were discriminated by the onset of active heartbeat and circulation (Harris 2012). Subsequently, the placentas with live embryos were quickly washed in sterile Tyrode's solution.

Histopathological observation of the LZ

For histological observation of the LZ, two placentas per dam were selected at random. The placentas were fixed in 0.1 M phosphate-buffered 2% paraformaldehyde and 1.25% glutaraldehyde (pH 7.4). Following fixation, the placentas were rinsed three times in phosphate buffer, postfixed for 2 h at 4°C in 0.1 M phosphate-buffered 2% osmium tetroxide and dehydrated in alcohol. The placentas were embedded in epoxy resin, and semithin sections (1 μm) were stained with toluidine blue.

For observation of vascular cast in the LZ, three pregnant mice in each group on day 13 of gestation were perfused for rinsing of the blood vessels with heparinized phosphate-buffered saline (PBS) (40°C) after anesthesia using sevoflurane. Subsequently, each mouse was perfused with Mercox II resin (Ladd Research, Williston, VT, USA). The placentas infused with resin were collected and polymerized for 2 h in an oven at 60°C. Polymerized placental tissues were corroded with 20% potassium hydroxide (KOH) in the oven at 60°C for 2 days. Vascular casts were rinsed with distilled water, and the sample was dried in a desiccator. The dried specimens were affixed onto aluminum stubs with double-sided tape, sputter-coated with palladium-gold and observed under a scanning electron microscope (Hitachi S-3500N, Japan).

As for the immunohistochemical observation of glucose transporter-1 (GLUT-1) in the LZ, two placentas per dam were selected at random. The placentas were fixed for 3 h in periodate lysine paraformaldehyde (PLP) at 4°C and washed in PBS. The placentas were embedded in paraffin and sectioned at 5 μm for immunohistochemical observation. The following antibody was used: rabbit monoclonal anti-glucose transporter (GLUT-1, 1:200; Abcam, Cambridge, UK). Secondary antibody was conjugated with Alexa 488 (1:200, Invitrogen, Carlsbad, CA, USA). Immunohistochemistry was performed as described previously (Komada et al. 2012).

Measurement of blood glucose levels in dams and fetuses

The blood glucose levels in five of the seven dams in each group and their fetuses on day 13 of gestation were measured directly by a glucose oxidase method using Glucose Pilot (Aventir Biotech, Carlsbad, CA, USA) in blood samples obtained from dam and fetus, according to the manufacturer's instructions. Fetal blood was collected in 10 μL heparinized capillary tubes from the severed end of the umbilicus (umbilical vein) by the method described by Renfree et al. (1975), and maternal blood was collected from the uterine artery. Each sample from five dams and 25 fetuses per group was measured.

Statistical analysis

Mean fetal weight, placental weight and glucose level were analyzed using Student's *t*-test. The frequency of resorbed embryos was compared by the Mann–Whitney *U*-test.

RESULTS AND DISCUSSION

Embryonic mortality and the weight of embryos and placentas

No adverse effects on the general conditions in maternal animals were observed in the DES group and the control group. A significant ($P < 0.01$) increase in embryonic mortality in terms of resorptions, in the DES group (70.8%) was detected compared with that in the controls (1.9%), and the rate was compatible with that (68.5%) observed in groups exposed to DES at 10 μg/kg per day in our previous study (Nagao et al. 2012). In addition, it was reported that the embryonic mortality after oral exposure to DES at 10 μg/kg per day on days 4 through 8 of gestation (around the time of implantation to chorioakkantoic fusion) was approximately 25% when examined on day 9 in mice (Nagao and Yoshimura 2009). Thus, it is suggested that many embryos of dams exposed to DES at this dose on days 4 through 8 proceeded to death during days 9 through 13 of gestation. The trophoblast undergoes extensive villous branching to create a densely packed structure called the LZ in the mouse placenta during days 9 through 13 (Rossant and Cross 2001).

It is well known that exposure to synthetic estrogens causes a high rate of embryonic death in rodents. The administration of estradiol benzoate (EB) and DES causes a high frequency of embryonic loss in rats and mice (Scott and Adejokun 1980; Sarkar et al. 1986; Matsuura et al. 2004; Furukawa et al. 2013). Embryonic weight in both sexes in the DES group (males, 0.179 ± 0.022 g; females, 0.176 ± 0.028 g) showed a tendency to decrease compared with that in the controls (males, 0.182 ± 0.026 g; females, 0.178 ± 0.036 g), and the embryonic weight/placental weight ratio in the DES group (males, 1.761 ± 0.112; females, 1.739 ± 0.185) was significantly ($P < 0.05$) decreased in both sexes compared with that in the controls (males, 1.929 ± 0.212; females, 1.904 ± 0.233), suggesting that oral DES dosing affects the intrauterine growth of embryos.

Histopathological changes of LZ

Light microscopy of the placenta clearly showed that the control placenta of a dam on day 13 of gestation had a rich villous network in the LZ and the group exposed to DES at 10 μg/kg per day showed the lack of a developing LZ (Fig. 1A,B), and evident dilatation of maternal blood space (Fig. 1C,D). For the ultrastructural observation of the LZ, the plexus vasculosus (PV) in the LZ of the DES group was thinner than that of the control group (Fig. 1E,F), although the branching structure in the DES group was well developed, similar to that in the controls. There were no distinct differences in the development of central arterial canal (CAC) between the DES group and the control group.

The LZ, where most gaseous and nutrient exchange occurs, was markedly underdeveloped by the early to middle gestational exposure to DES. Matsuura et al. (2004) also demonstrated that the rat placenta exposed prenatally to EB lacked the developing LZ, and extensive maternal blood space was evident and the density of fetal blood vessels was significantly decreased. Furukawa et al. (2013) showed that cystic dilatation of the sinusoid was observed in the LZ of rats exposed to EB. Taking these findings together, it is suggested that disturbance of the villous network of the LZ resulting from reduced villigenesis might be associated with decreases in the physiological function of the placenta.

The cellular uptake of nutrient, glucose, across the plasma membrane is mediated by integral membrane proteins, GLUTs. Thus, the expression level of the GLUT-1 protein in the LZ of the placenta was investigated by immunohistochemical techniques. GLUT-1-positive cells in the LZ of the DES group were markedly decreased in comparison with those of the controls when evaluated on day 13 of gestation (Fig. 1G,H). Decreased GLUT-1 in the LZ of the DES group may be related to underdevelopment of the LZ, in particular, lack of a developing PV. The expression of two GLUT isoforms has been demonstrated in mammalian placenta, namely, GLUT-1 and GLUT-3 (Zhou and Bondy 1993; Devaskar et al. 1994; Barros et al. 1995). The predominant form expressed in the placenta during early