with the two antiandrogens. Rat testicular protein extracts of control and treated animals were analysed by 2-D gel electrophoresis coupled with MALDI-TOF. This technique can detect differentially expressed proteins between control and treated testicular samples. Of the total protein population observed, we identified a number of proteins which are involved in several cellular processes: detoxification mechanism, apoptosis, translation, metabolism, signaling pathway, immune response, chaperones. Further investigations will be necessary to find the relevant protein which ultimately contribute to the cellular changes observed in the testis following treatment with antiandrogens.

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EFFECT OF BISPHENOL A ON CENTRAL NERVOUS SYSTEMS

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Bisphenol A (BPA) is a monomer of polycarbonate plastics and used in the lining of food cans and dental sealants. BPA is suspected to be one of endocrine disrupting chemicals (EDCs) due to its acting as an agonist for the estrogen receptors. Although many investigations have suggested that some chemicals disrupt reproductive systems, the effects on central nervous systems are not well known. We previously found that prenatal and lactational exposure of BPA reduced dopamine (DA) contents in mice. Since BPA was suspected to affect central nervous system through dopaminergic neuron, we convinced that the novel receptor of BPA presented in synaptosomal membrane fraction in the brain. In this study, we isolated and purified the BPA binding protein from rat synaptosomes using BPA-Sepharose affinity column chromatography technique. N-terminal sequence of the purified protein was identical with protein disulfide isomerase (PDI) which was also known as thyroid hormone binding protein. The competition binding assay was performed to elucidate possible molecular mechanism of the BPA action on thyroid hormone binding. BPA inhibited the binding of 3, 3', 5-triiodothyronine (T_3) to PDI with an $IC_{50} = 22.1$ μM which was 10-100 fold lower affinity than T_3 ($IC_{50} = 0.79$ μM). This effective potency of BPA suggest interfere the thyroid hormone action via PDI. We also examined the displacing activity of T_3 from PDI by other chemicals which were suspected as EDCs. The binding of T_3 to PDI was inhibited by 4-octylphenol, 4-nonylphenol, pentachlorophenol, 2, 4-dichlorophenol, tetrabromobisphenol A, and tetrachlorobisphenol A. BPA and these chemicals mimic the thyroid hormone action by competing with thyroid hormone bound to PDI.

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ENDOCRINE DISRUPTING CHEMICALS - OLD AND NEW - REASONS FOR CONTINUED PUBLIC HEALTH INTERVENTION

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The United Nations Environmental Programme indicates the production of new chemicals are increasing every year on a global scale. There are approximately 50,000 to 100,000 chemicals being produced commercially. The potential human health threat from exposure to these chemicals is unknown. Chemicals such as polybrominated diphenyls ethers (PBDEs), perfluorosulfonates (PFOS), chlorinated naphthalenes, perchlorates and others are new threats to the environment and human health. All of these chemicals have been found in the environment, and have been shown to bioaccumulate in humans. For example, in the U.S. Great Lakes basin PBDEs have been found in air, sediment, biota, wildlife, human fatty tissue, blood serum, and breast milk. The chemical structure of PBDEs is similar to PCBs and therefore may cause neurological effects from exposure. Initial animal studies indicate PBDEs can cause neurologic effects from exposure. Perchlorates are colorless and ordorless salts used in explosives and rocket motors and persist in water and soil. Perchlorates affect the ability of the thyroid gland to make hormones that regulate many body functions and development. This presentation will discuss these new emerging chemicals of concern and provide new information about health effects from the old persistent organic pollutants (POPs), such as polychlorinated biphenyls.

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SPECIES DIFFERENCES IN THE EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORα (PPARα)-RELATED ENZYMES BY DI (2ETHYLHEXYL) PHTHALATE (DEHP)

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Male CD-1 mice, SD rats and marmosets (*Callithrix jacchus*) were treated with 0, 1.25 and 2.5 mmol/kg DEHP (for mice and rats) for two weeks, and with 0, 0.25, 1.25 and 6.25 mmol/kg DEHP (for marmosets) for fifteen months by gavage. On the day after the last dose (18-20 hours later), all animals were killed by CO_2 asphyxiation. The induction of PPARa-related peroxisomal and mitochondrial enzymes were investigated.

Immunoblotting analysis clearly showed a band detected by anti-CYP4A in the liver from rats, but not in the livers of mice or marmosets. The PT and PH detected by anti-PT and anti-PH in the livers from mice and rats, but not in livers from marmosets, are induced by exposure to DEHP. These enzyme inductions were most prominent in livers from rats, followed by mice. MCAD and VLCAD detected by anti-MCAD, and anti-VLCAD were also induced by DEHP exposure in mice and rats, but to a lesser extent compared with those in the peroxisomal enzymes. In contrast, these enzymes could not be induced in the liver from marmosets.

MCAD-mRNA levels were induced in the livers from mice and rats exposed to DEHP, but that from marmosets tended to decrease after exposed to 1.25 and 6.25 mmol/kg DEHP. PT-mRNA levels were strongly induced in the liver from rats exposed to DEHP, following by levels from mice, whereas those of marmosets decreased after exposure to 1.25 or 6.25 mmol/kg in the same manner as MCAD. These results are quite similar to changes in the respective proteins. Thus, we concluded that there are also species differences in DEHP-induced PPARα-related enzymes; species differences in induction of peroxisomal enzymes are most prominent.

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A STUDY ON THE DEVELOPMENTAL TOXICITY OF ESTROGENIC CHEMICALS IN EARLY LIFE STAGE OF FISH

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The endocrine system plays an essential role in the development, growth, reproduction, and behavior of human beings and animals. A balance of steroid hormones in organism is essential to maintain normal gonadal differentiation and morphological development. Some environmental chemicals have been reported to possess the hormone-like activities. Thus they may result in reproductive disturbances and affect the developmental process.

The purpose of this study was to determine the effects of nonylphenol, bisphenol A and diethylstilbestrol (DES), which were known to have estrogenic activity, on the morphological development of Japanese med-

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Original

Di-n-butyl Phthalate is Toxic to the Male Reproductive System and Its Toxicity is Enhanced by Thioacetamide Induced Liver Injury

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Abstract: The modifying effects of liver injury on male reproductive organ toxicity of di-n-butyl phthalate (DBP) were studied using male F344 rats. Seventy-two male animals, aged 10 weeks at the commencement, were divided into 8 groups of 9 rats each. Groups 1 to 4 were given 200 mg/kg bw of thioacetamide (TAA) intraperitoneally 3 times / week, while Groups 5 to 8 were injected with the PBS vehicle only. From 1 week after the start, groups 1 and 5, 2 and 6, and 3 and 7 were given daily 500, 125, 31.25 mg/kg bw of DBP, respectively (23 times) for 4 weeks, while groups 4 and 8 served as vehicle controls. As the result of our treatment, liver injury was induced in the animals, and the liver injury was confirmed by histopathological findings and serum transaminase levels at sacrifice. Enhancement of rat male reproductive organ toxicity of DBP under liver injury status was also seen. The relative organ weights of prostate, bilateral seminal vesicles, bilateral epididymides and bilateral testes, and the sperm number and motility rate were decreased. Also the sperm abnormality rate was increased under liver injury. Furthermore histopathological abnormality of the testis, such as absence of germ cells and the presence of Sertoli cells only was seen in the rats treated with TAA and high dose of DBP, however neither the serum nor testicular testosterone levels varied in the present study. The sperm number and relative weight of bilateral epididymides were decreased by TAA treatment only, unrelated to DBP treatment. These facts suggest that liver injury can not only enhance the rat male reproductive organ toxicity of DBP, but also induce such toxicity by itself. (J Toxicol Pathol 2004; 17: 177–185)

Key words: DBP, male reproductive organ toxicity, F344 rat, TAA, liver injury

Introduction

Recently, there has been increasing concern about the effects of environmental chemicals on human reproduction. A large number of chemical agents have been reported to affect reproduction in laboratory animals and some of them have been demonstrated to also influence humans[1]. For example, di-n-butyl phtalate (DBP), a widely used industrial solvent and plasticizer, has been recognized as a ubiquitous contaminant of the environment and is suspected to be an endocrine disrupter[2]. Blount *et al.*[3,4] noted several phthalate ester metabolites at the ppm level in the urine of people in the general population and found that mono-n-butyl phthalate, the major active metabolite of DBP, demonstrated its highest concentrations in women of childbearing age in the 20-40 year age group. They did not report exposure levels resulting in these urinary concentrations, but others[5] have used the data and estimated with reasonable assumptions that for the average female, the maximum exposure to DBP via the oral route would be ~113 μg/kg/day. This value is more than two orders of magnitude lower than the NOAEL (50 mg/kg/day) for DBP from animal studies[6,7]. In Japan, however, food contamination with DBP contained in plastic gloves has recently become a cause for concern.

DBP has been shown to exert hepatotoxic, cytotoxic, mutagenic effects and produce renal, pulmonary and reproductive dysfunction in experimental animals[8]. It is doubtful whether the liver would be a target of this chemical in humans, because the effects are due to species specific actions on peroxisomes[9]. Much attention, however, has been paid to its male reproductive organ toxicity. At doses ≥ 1 g/kg bw/day, DBP is a testicular toxicant in rodents, causing seminiferous tubule degeneration, with young animals being more susceptible than adults to this toxic effect[10]. At lower dose levels (≥ 250 mg/kg/day), when administered during the major period of male reproductive organogenesis in the

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rat (gestation days 12–21), DBP blocks male reproductive development, resulting in feminization of male pups, malformation of the male reproductive organs, seminiferous tubule degeneration, and Leydig cell hyperplasia and adenomas[11].

DBP is hydrolyzed by lipase to mono-n-butyl phthalate, which is thought to be the active form[12]. This material is then oxidized in the liver and excreted into the urine in experimental animals[12]. It is thought that the mechanism of testicular atrophy induced by DBP in rats might be associated with iron release from hemoglobin and/or transferrin in the liver[13]. Liver dysfunction might delay the metabolism of DBP and thus enhance its male organ reproductive toxicity, although there have hitherto been no relevant experimental data in support of this supposition. In the present study, therefore, the modifying effects of liver injury on male reproductive organ toxicity of DBP were studied using F344 rats.

Thioacetamide (TAA) is a thiono-sulfur-containing compound which is widely used as an organic solvent in the leather, textile and paper industries, and as an accelerator in the vulcanization of rubber and as a stabilizer of motor fuel. It also causes liver damage and hepatoneoplasia. Oren R *et al.*[14] reported that oral administration of TAA (0.03% in tap water) for 12 weeks induced severe distortion of the liver architecture with generation of nodules surrounded by fibrotic septa in livers of female Wistar rats. Similar results were obtained in a study using male F344 rats in our laboratory (unpublished data). Furthermore, Reif S. *et al.*[15] reported that liver cirrhosis was induced in male Wistar rats by intraperitoneal injection of TAA, 200 mg/kg twice weekly for 12 weeks. After establishment of a suitable protocol to induce liver injury by TAA, we here investigated its modifying effects on the male reproductive organ toxicity of DBP in F344 rats in the present study.

Materials and Methods

Animals

Seventy-two male F344/DuCrj rats were obtained at 9 weeks of age from Charles River Japan Inc. (Hino, Shiga). They were housed 3 animals per stainless steel cage with paper chips for bedding and placed in a controlled environment, air conditioned at 24 ± 2°C, with a relative humidity of 55 ± 20% and a 12h/12h light-dark cycle. Food (CE-2 pellet diet; Clea Japan Inc., Tokyo) and water were available *ad libitum* throughout the period of the experiment. Experimentation was started after 1 week of acclimation and was approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

Chemicals

DBP was purchased from Tokyo Kasei Co. Ltd. (Tokyo) and TAA from Sigma Chemical Co. (St. Louis, MO, USA). Medium 199 with Hank's salt and L-glutamine was obtained from Gibco Co. (NY, USA) and 1.0% w/v bovine serum albumin from Intergen Co. (NY, USA). Corn oil was purchased from Wako Pure Chemical Industries Ltd. (Osaka). All chemicals used were of the highest purity commercially available.

Experimental design

Srivastava *et al.*[16] reported that DBP treatment of Wistar albino rats caused decrease of the sperm count and disorganization of seminiferous tubules at a dose of 500 mg/kg body weight, but no marked histopathological changes at 250 mg/kg body weight after 15 days of oral administration. Therefore the high dose of DBP used in our study was set as 500 mg/kg body weight. At 10 weeks of age, the rats were divided into 8 groups (9 rats each) (Fig. 1). Animals in groups 1 to 4 were given 200 mg/kg body weight of TAA with 3 ml of PBS from the commencement of the experiment to week 5 (ip., 3 times a week). Groups 5 to 8 were injected with 3 ml of PBS only as vehicle controls. From 1 week after the start of the experiment, groups 1 and 5 were given 500 mg/kg body weight of DBP daily with 3 ml of corn oil (ig. 5 times a week). Groups 2 and 6 similarly received 125 mg/kg body weight and groups 3 and 7 were administered 31.25 mg/kg body weight. Groups 4 and 8 were given corn oil only. The doses of TAA used in the present study were chosen based on the results of a preliminary study (unpublished data). Body weights were measured every other day during week 1 and every day during weeks 2 to 5. Food consumption and water intake were measured once weekly. All rats were sacrificed under ether anesthesia at week 5, and liver, kidneys, prostate (ventral, dorsolateral), seminal vesicles with coagulate glands, testes and epididymides were excised and weighed. Before sacrifice, blood samples were taken for measurement of serum transaminase and testosterone levels, and frozen samples of left testes and epididymides were also taken and used for measurement of testosterone levels by radioimmunoassay.

Tissue processing and morphological examination

The right testis and epididymis of each animal were fixed in GFA (glutaraldehide, formalin and acetic acid) solution for 1 day and replaced in 10% phosphate-buffered formalin solution. Slices 2–3 mm thick were taken from three lobes of livers and fixed with samples of kidneys and prostate fixed in 10% phospate-buffered formalin. The tissues were routinely processed for embedding in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin (H and E) for histopathological examination. Liver sections were also processed for Azan and Mallory (A and M) staining.

Sperm analysis

Six rats from each group were used for sperm analysis.

Sperm counts: The left epididymis resected at sacrifice were homogenized and sonicated with 10 ml water, and sperm heads were counted using a hemocytometer under a light microscope and recorded. The densities were calculated on the basis of the dilution rate.

Sperm motility tests: One droplet of sperm was

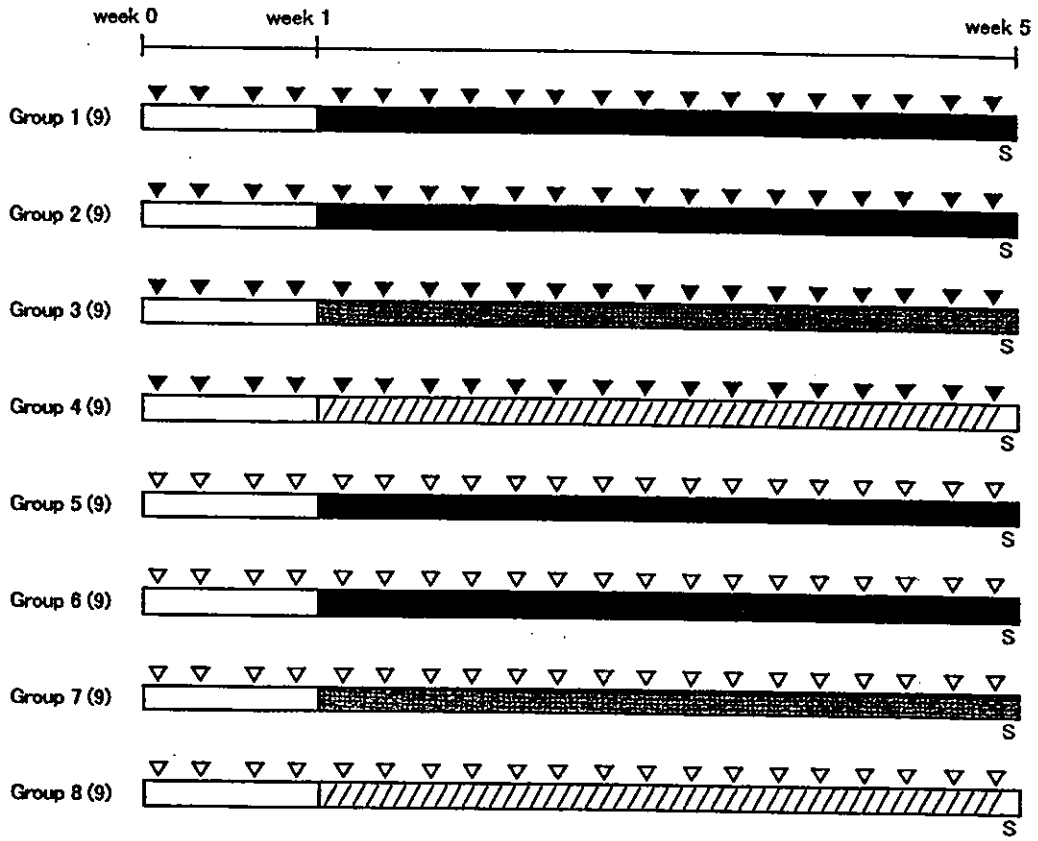

Fig. 1. The experimental protocol. Animals were 10-week-old male Fischer 344 rats. ▼; TAA, 200 mg/kg. Ip 3 times per week. ▽; vehicle (PBS) only ip 3 times per week. Black, Dark gray and Light gray; DBP at 500, 125 and 31.25 mg/kg/day ig. with 0.4 ml of corn oil once a day for 28 days. Cross hatched; vehicle (corn oil) only ig. once a day for 28 days. S; time points of sacrifice. Number of animals examined is shown in parentheses.

collected from a puncture of the left caudal epididymis, and introduced into 2 ml of sperm suspension medium [Medium 199 with Hank's salt and L-glutamine (Gibco Co. NY, USA)] supplemented with 1.0% w/v bovine serum albumin (Intergen Co. NY, USA) maintained at 37°C. The sperm suspensions were plated at 37°C for 5 min so that sperm could diffuse completely in the media, then examined under an optical microscope and the numbers of motile sperm were counted for a total of 100 sperm. The % motility was calculated from the data using the following formula: number of motile sperm × 100 / total number of observed sperm.

Sperm morphology: The suspensions for sperm motility analysis were also used for counts of abnormal sperm in 200 sperm. The samples were dried on hot plate and fixed for observation. Abnormal shapes were defined as follows: no head, no hook, banana shape head, excessive hook, pin head, two head, amorphous, two tails and short tail.

Statistical analysis

Statistical analyses were performed with Stat-View software for Macintosh computers. Student's *t* test was used for testing differences in body weights, relative organ weights, food consumption, water intake, sperm numbers, sperm motility rates, sperm abnormality rates, serum transaminase levels and serum and testicular testosterone levels between liver injury induced rats and rat without liver injury. For testing the dose effect of DBP as significance, the post hoc test was used. Values of $p<0.05$ and <0.01 were considered as significant difference.

Results

Body weight, water intake and food consumption

Growth retardation in groups 1 to 4 was observed, compared to the corresponding groups of rats without liver injury induction, groups 5 to 8 (Table 1), and was also slight but significant in group 5 compared to group 8. Furthermore, food consumption was significantly less than in each of the corresponding control groups (groups 5 to 8) (Table 1). In contrast, water consumption was similar in all groups (Table 1).

Relative organ weights

The relative weights of liver, bilateral kidneys, seminal

Table 1. Body Weight, Water Intake and Food Consumption

Group	Treatment		Examined No. of animals	Body weight (g)		Food consumption (g/day/rat) week 1–4	Water intake (ml/day/rat) week 1–4
	TAA	DBP		Initial	Final		
1	+	500 mg/kg/day ig.	9	224 ± 5	206 ± 7[a]	8.9[a]	23
2	+	125 mg/kg/day ig.	9	222 ± 6	211 ± 11[a]	8.4[a]	20
3	+	31.25 mg/kg/day ig.	9	223 ± 5	207 ± 10[a]	8.4[a]	21
4	+	corn oil only	9	224 ± 5	206 ± 16[a]	9.5[a]	22
5	–	500 mg/kg/day ig.	9	222 ± 6	270 ± 13[b]	14	22
6	–	125 mg/kg/day ig.	9	223 ± 3	276 ± 9	14	22
7	–	31.25 mg/kg/day ig.	9	223 ± 7	278 ± 9	14	21
8	–	corn oil only	9	223 ± 6	285 ± 9	15	21

Data are mean ± SD values.
ig.; intragastrial injection. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$. b; Significantly different from non-TAA treatment and corn oil only ig. group. (group 8), $p < 0.05$.

Table 2. Relative Organ Weight of Animals

Group	Treatment		Examined No. of animals	Liver	Kidneys	Testes	Epididymides	Prostate		
	TAA	DBP						ventral lobe	dorsolateral lobe	seminal vesicles + coagulate glands
				(%)	(%)	(%)	(%)	(%)	(%)	(%)
1	+	500 mg/kg/day ig.	9	4.97 ± 0.28	0.85 ± 0.08[a]	0.39 ± 0.04[a]	0.18 ± 0.03[a,b]	0.06 ± 0.03[d]	0.04 ± 0.02[a]	0.06 ± 0.02[d]
2	+	125 mg/kg/day ig.	9	4.77 ± 0.22[a]	0.82 ± 0.05[a]	1.06 ± 0.13	0.28 ± 0.03	0.06 ± 0.03[d]	0.07 ± 0.03[d]	0.07 ± 0.02[d]
3	+	31.25 mg/kg/day ig.	9	5.03 ± 0.40[a]	0.83 ± 0.06[a]	1.11 ± 0.08[a]	0.30 ± 0.04	0.06 ± 0.02[d]	0.05 ± 0.02[d]	0.07 ± 0.01[d]
4	+	corn oil only	9	4.85 ± 0.57[a]	0.82 ± 0.08[a]	1.08 ± 0.11	0.28 ± 0.04	0.08 ± 0.04[d]	0.06 ± 0.02[a]	0.07 ± 0.01[d]
5	–	500 mg/kg/day ig.	9	4.40 ± 0.23[c]	0.71 ± 0.03[c]	1.01 ± 0.07	0.30 ± 0.02	0.14 ± 0.04	0.09 ± 0.04	0.28 ± 0.05
6	–	125 mg/kg/day ig.	9	3.72 ± 0.23[c]	0.66 ± 0.02[c]	0.99 ± 0.13	0.29 ± 0.03	0.15 ± 0.02	0.11 ± 0.03	0.29 ± 0.02
7	–	31.25 mg/kg/day ig.	9	3.50 ± 0.12	0.64 ± 0.02	1.05 ± 0.04	0.30 ± 0.02	0.14 ± 0.02	0.11 ± 0.02	0.29 ± 0.04
8	–	corn oil only	9	3.33 ± 0.07	0.63 ± 0.02	1.02 ± 0.10	0.39 ± 0.02	0.15 ± 0.03	0.11 ± 0.05	0.31 ± 0.02

Data are mean ± SD values.
ig.; intragastrial injection. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$. b; Significantly different from TAA treatment and corn oil only ig. group (group 4), $p < 0.05$. c; Significantly different from non-TAA treatment and corn oil only ig. group (group 8), $p < 0.05$. d; Significantly different from each corresponding non-TAA treatment group. (group 5 to 8), $p < 0.01$.

vesicles with coagulate glands in groups 1 to 4 were significantly larger than those of each corresponding control group (groups 5 to 8), except for the liver value in group 1 (Table 2). The relative weights of bilateral epididymides in group 1 were significantly lower than in group 4, and the relative weights of the bilateral testes of groups 1 and 3 were significantly lower than those of the corresponding groups of rats without liver injury induction, groups 5 and 7 (Table 2).

The results of sperm analysis

The results of sperm analysis are summarized in Table 3. Sperm numbers of groups 1 to 4 were significantly lower than those of each corresponding control group (groups 5 to 8), and the sperm number of group 1 was also significantly lower than that of group 4 (Table 3). Furthermore, the sperm number of group 6 was significantly lower than that of group 8 (Table 3). The sperm motility rate for group 1 was also significantly lower than those for both groups 4 and 5 (Table 3).

Furthermore, sperm abnormality rates of groups 2 to 4 were significantly larger than those of each corresponding control group (6 to 8) (Table 3), and those of groups 5 to 7 were also significantly higher than that of group 8 (Table 3).

Serum transaminase levels

Both serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly elevated in groups 1 to 4 compared to each corresponding non-TAA treatment group (5 to 8) (Table 4). However, no differences in serum transaminase levels were apparent between any of the liver injury-induced groups (groups 1 to 4) and their counterparts without liver injury induction (groups 5 to 8).

Serum and tissue testosterone levels

There was no specific variation in either serum or tissue testosterone levels in TAA or DBP treated animals (Table 5).

Histopathological findings

On histopathological examination, fibrosis, increased

Table 3. The Data of Sperm Analyses of Animals

Group	Treatment		Examined No. of animals	Sperm number (/ml)	Sperm motility rate (%)	Sperm abnormality rate (%)
	TAA	DBP				
				$\times 100^2$		
1	+	500 mg/kg/day ig.	9	$45 \pm 70^{a,b}$	$2.9 \pm 4.7^{a,b}$	23.2 ± 10.9
2	+	125 mg/kg/day ig.	9	1083 ± 368^{a}	57.6 ± 30.1	30.0 ± 9.8^{a}
3	+	31.25 mg/kg/day ig.	9	1245 ± 373^{a}	75.4 ± 20.3	20.9 ± 10.4^{a}
4	+	corn oil only	9	1452 ± 533^{a}	78.5 ± 23.3	29.1 ± 10.4^{a}
5	−	500 mg/kg/day ig.	9	2748 ± 683	82.4 ± 5.8	17.7 ± 7.8^{c}
6	−	125 mg/kg/day ig.	9	2067 ± 287^{c}	85.1 ± 9.1	16.4 ± 2.6^{c}
7	−	31.25 mg/kg/day ig.	9	3093 ± 713	86.1 ± 6.4	9.3 ± 4.3^{c}
8	−	corn oil only	9	3172 ± 479	74.2 ± 36.6	2.4 ± 1.1

Data are mean ± SD values.
ig.; intragastrial injection. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$. b; Significantly different from TAA treatment and corn oil only ig. group (group 4), $p < 0.05$. c; Significantly different from non-TAA treatment and corn oil only ig. group (group 8), $p < 0.05$.

Table 4. Serum Transaminase Levels of Animals

Group	Treatment		Examined No. of animals	Serum transaminase level (IU/L/37°C)	
	TAA	DBP		AST	ALT
1	+	500 mg/kg/day ig.	9	258 ± 89^{a}	$136 \pm 43^{a,b}$
2	+	125 mg/kg/day ig.	9	191 ± 47^{a}	90 ± 17^{a}
3	+	31.25 mg/kg/day ig.	9	132 ± 18^{a}	64 ± 12^{a}
4	+	corn oil only	9	192 ± 63^{a}	88 ± 30^{a}
5	−	500 mg/kg/day ig.	9	84 ± 10	41 ± 5
6	−	125 mg/kg/day ig.	9	75 ± 10	42 ± 4
7	−	31.25 mg/kg/day ig.	9	77 ± 6	47 ± 4
8	−	corn oil only	9	84 ± 10	45 ± 4

Data are mean ± SD values.
ig.; intragastrial injection. AST; aspartate aminotransferase. ALT; alanine aminotransferase. a; Significantly different from each corresponding non-TAA treatment groups (group 5 to 8), $p < 0.05$. b; Significantly different from TAA treatment and corn oil only ig. group (group 4), $p < 0.05$.

Table 5. Serum and Testicular Testosterone Levels of Animals

Group	Treatment		Examined No. of animals	Serum Testosterone Level (ng/ml)	Examined No. of animals	Testicular Testosterone Level (ng/ml)
	TAA	DBP				
1	+	500 mg/kg/day ig.	9	0.74 ± 0.78	5	22.8 ± 11.5
2	+	125 mg/kg/day ig.	7	1.29 ± 1.51	5	42.7 ± 31.5
3	+	31.25 mg/kg/day ig.	7	1.62 ± 1.76	4	37.1 ± 25.2
4	+	corn oil only	9	1.09 ± 1.43	5	13.5 ± 10.8
5	−	500 mg/kg/day ig.	8	1.71 ± 1.70	5	29.1 ± 16.5
6	−	125 mg/kg/day ig.	9	3.16 ± 4.62	5	24.9 ± 12.2
7	−	31.25 mg/kg/day ig.	9	1.61 ± 1.21	5	24.9 ± 10.7
8	−	corn oil only	9	1.36 ± 1.39	5	11.7 ± 4.2

Data are mean ± SD values.
ig.; intragastrial injection.

area of Glisson's sheath, pigmentation and infiltration of inflammatory cells were seen in the liver samples from groups 1 to 4 (Figs. 2A, 2C). There was no such evidence of liver injury in groups 5 to 8 (Figs. 2B, 2D).

In the testis and epididymis, findings pointing to male reproductive organ toxicity were seen only in group 1; seminiferous tubules showed severe atrophic changes and the normal architecture was distorted and disorganized (Fig. 3A), and some epithelial cells of the ducts in the epididymis showed cells shed from the seminiferous tubules in the

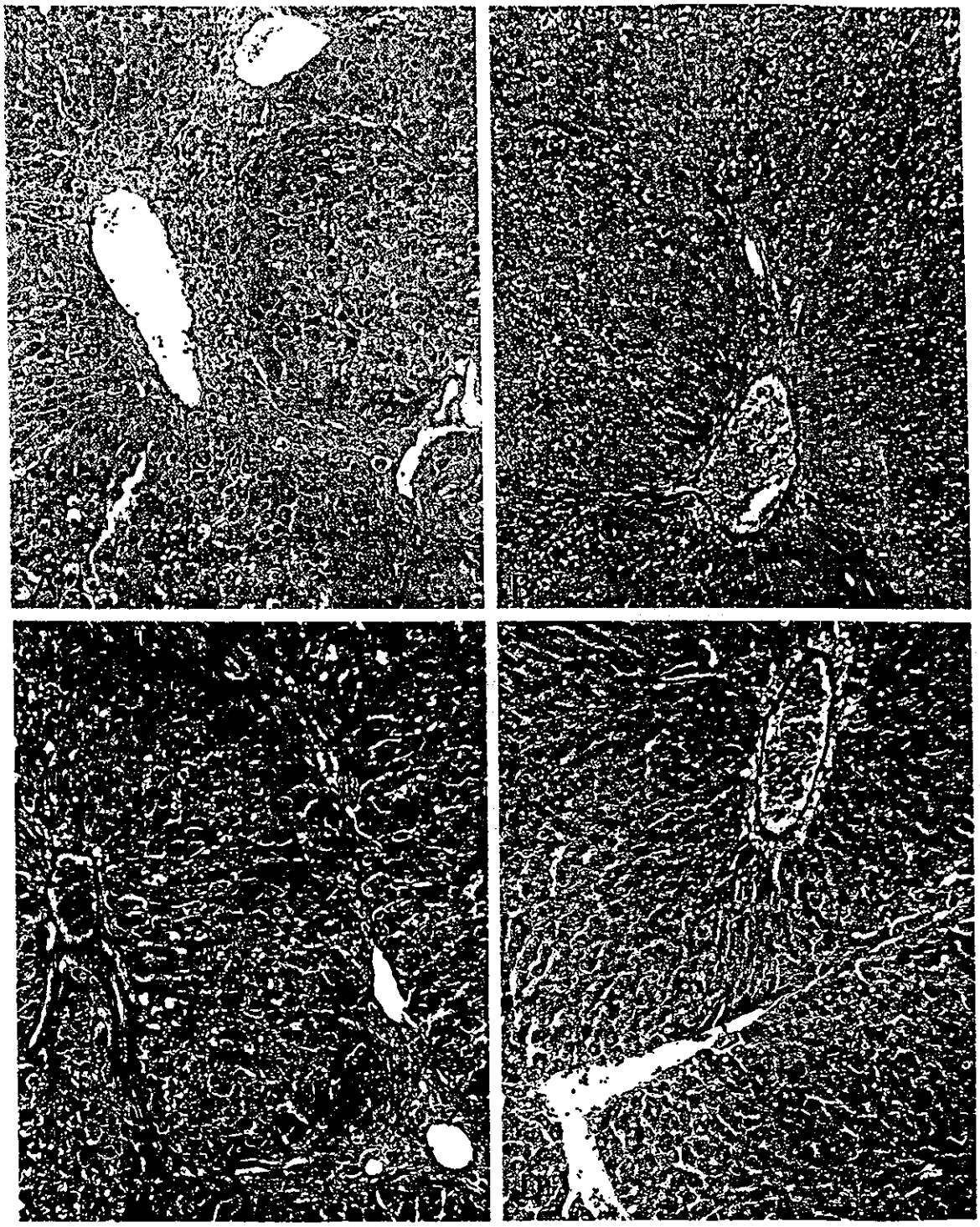

Fig. 2. Histopathological features of liver sections. Note fibrosis, increased area of Glisson's sheath, infiltration of inflammatory cells and pigmentation in a liver sample from group 1, in contrast to the control case (group 8). (A, B; H-E staining, × 200. C, D; A-M staining, × 200.).

lumina (Fig. 3C). Atrophic changes in testis were very mild in groups 2 to 7, and testes of group 8 demonstrated normal spermatogenesis (Fig. 3B) with no specific alteration evident in the epididymis (Fig. 3D).

Atrophic changes were apparent in prostate samples of liver injury-induced rats, but not in those of rats without liver injury induction.

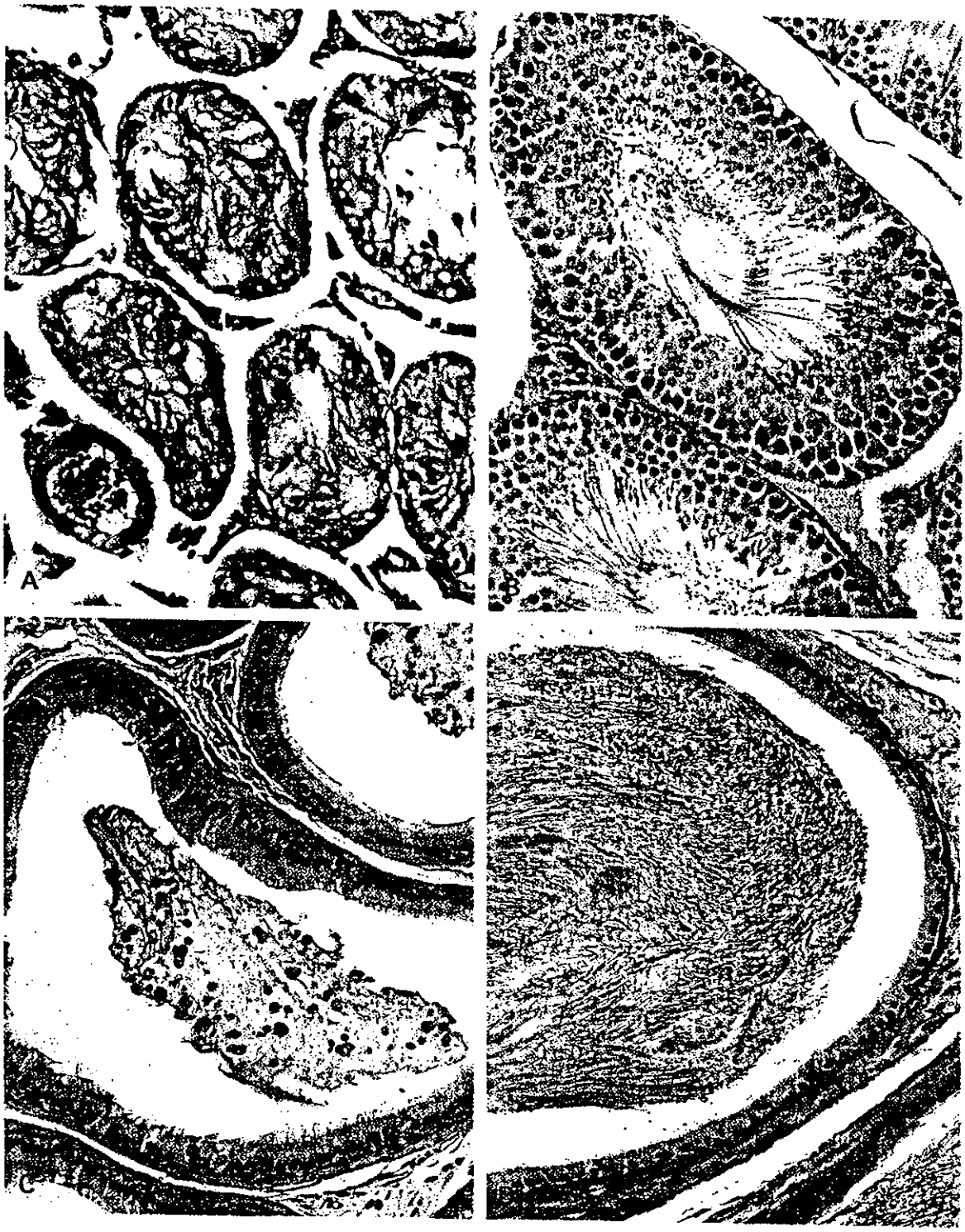

Fig. 3. Histopathological examinations of testis and epidydimis sections. Note evidence of male reproductive organ toxicity in the TAA treatment and DBP high dose group (group 1); seminiferous tubules show severe atrophic changes and the normal architecture is disturbed (A; H-E staining, × 200.), some of the epithelial cells of the ducts in an epididymis showing cells shed from seminiferous tubules evident in the lumen (B; H-E staining, × 200.). A testis of the non-treatment group 8 shows normal spermatogenesis (C; H-E staining, × 200.) and no specific alteration in the epididymis (D; H-E staining, × 200.).

Discussion

The present study clearly showed that liver injury induced by TAA enhances the toxicity of DBP in the male sexual organs. Furthermore, weak but significant rat male reproductive organ toxicity could be seen in the sperm numbers and sperm abnormality rate in TAA -administered rats without DBP. Whether this is direct or indirect remains to be clarified.

Male reproductive organ toxicity of DBP was limited to increased sperm abnormalities in rats without liver injury induction, whereas the sperm number, motility rate and relative organ weights of testes and epididymis were all altered in liver injury-induced rats.

The sperm number of 125 mg/kg/day DBP-treated rats was significantly low compared to that of control rats. Although this result is more sensitive to the toxicity of DBP than the results of our histopathological examination, there was no dose-response. Thus, it was considered to be incidental.

The relative liver weights of the 500 and 125 mg/kg/day DBP-treated rats were significantly higher than those of control rats, and this was probably due to the hepatotoxic effect of DBP.

The relative kidney weights of the 500 and 125 mg/kg/day DBP-treated rats were significantly higher than those of control rats, and the relative kidney weights of the liver injury-induced rats were also significantly higher than those of rats without liver injury induction. These findings were probably due to the relative low body weights of the treated animals.

In the present study, liver injury induced by TAA showed some level of rat male reproductive toxicity including sperm counts and sperm abnormality rate, suggesting that TAA might modify hepatic clearance of steroid hormones via liver injury. It is indeed well known that patients with advanced liver disease show testicular atrophy and morphological and morphometric changes in alcoholic and nonalcoholic liver disease in humans have been described by several authors[17–20]. A rat model for alchohol-induced gonadal atrophy has also been reported[21]. In fact, hepatic cirrhosis is one of the classical conditions known to have a profound adverse effect on male reproductive function[22], and the combination of testicular failure and features of feminization is well-known[23–26]. Hypogonadism and feminization are not confined to alcoholic cirrhosis and may accompany other types of cirrhosis[23,24], and milder forms may occur with non-cirrhotic liver disease[23]. It would thus appear that the TAA influence is likely to be indirect via its effects on liver function. However, the hypothesis that reduced hepatic clearance of estrogen results in elevated blood levels and secondary suppression of gonadotropin secretion and testicular function has been disproved[24,25], and it is now recognized that there is mainly a primary testicular disorder with elevated gonadtropin levels and reduced testosterone production[24].

It has previously been reported that phthalate esters show no direct effect on the prostate[2], however, atrophic changes of this organ were seen in our animals, and it is assumed that this finding was a result of the liver injury.

It has been not clarified how DBP induces male reproductive organ toxicity in animals, although anti-androgenic effects have been demonstrated in animal studies using both sexes of rats[2]. Mylchreest E *et al.*[12] reported that DBP treatment caused a marked decrease in testosterone levels in the fetal testes of Sprague-Dawley rats.

Unexpectedly, there was no significant difference of the serum and testicular testosterone levels in our rats, though apparent anti-androgenic findings were observed. The small sample number might be the cause of this contradiction.

The sperm number in the epididymis of rats treated with both TAA and 125 or 31.5 mg/kg of DBP were diminished, but the relative epididymis weights of these rats were not diminished. We can offer no explanation for this apparent contradiction.

It has been reported that mono-n-butyl phthalate, the major metabolite of DBP, disrupts Sertoli cell-germ cell interactions *in vitro*[26,27]. Furthermore, Mylchreest E *et al.*[12] reported that DBP treatment did not induce atrophy of the prostate, the major target organ of flutamide, a well-known androgen receptor antagonist. Gray *et al.*[28] also reported the lack of interaction of DBP and mono-n-butyl phthalate with androgen receptors *in vitro*. Thus, Koizumi M *et al.*[2] concluded that the mechanism of action of DBP is not via androgen receptors. Estrogenic effects of DBP *in vitro* have been described[29,30], but Koizumi M *et al.*[2] could not confirm these *in vivo*. Further examination is needed to clarify the mechanisms underlying the results of the present study.

In conclusion, the results of the present study suggest that liver injury enhances the rat male reproductive organ toxicity of DBP, and this phenomenon might be caused by the inhibition of its metabolism in the liver and its accumulation. Furthermore, this implies that other endocrine disrupters which are detoxified in the liver will be also having their effects enhanced under liver dysfunction. The effects of DBP on humans should not be underestimated and further examination of this issue is needed.

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Novel type of $G_{q/11}$ protein-coupled neurosteroid receptor sensitive to endocrine disrupting chemicals in mast cell line (RBL-2H3)

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Running title: $G_{q/11}$ protein-coupled neurosteroid receptor

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Summary

1. Agonistic neurosteroids, including pregnenolone, dehydroepiandrosterone and its sulfate (DHEAS), caused rapid degranulation in measurements of -hexosaminidase (-HEX) release from a mast cell line, RBL-2H3. This degranulation was blocked by BSA-conjugated progesterone (PROG-BSA) or 17 -estradiol, both of which are antagonistic neurosteroids.
2. DHEAS-induced -HEX release was blocked by U-73122 or xestospongin C, but not by PTX or EGTA. DHEAS-induced -HEX release was also abolished by $G_{q/11}$-AS, but not by $G_{q/11}$-MS. Pharmacological analyses revealed that the neurosteroids stimulated a putative membrane receptor through activation of the novel $G_{q/11}$ and phospholipase C.
3. While representative endocrine-disrupting chemicals (EDCs) did not show any degranulation or nocifensive actions by themselves, they blocked the DHEAS-induced degranulation.
4. The binding of a PROG-BSA-fluorescein isothiocyanate conjugate (PROG-BSA-FITC) to cells was inhibited by neurosteroids and EDCs.
5. In the algogenic-induced biting and licking responses test, DHEAS caused agonistic nocifensive actions in a dose-dependent manner between 1 and 10 fmol (i.pl.). DHEAS-induced nocifensive actions were abolished by PROG-BSA or nonylphenol.
6. Taken together, these results suggest that a $G_{q/11}$-coupled neurosteroid receptor may regulate the neuroimmunological activity related to sensory stimulation and that some EDCs have antagonistic actions for this receptor.

Keywords. neurosteroid, endocrine-disrupting chemical, GPCR, mast cell, -hexosaminidase release

Abbreviations. DHEAS, dehydroepiandrosterone sulfate; PREGS, pregnenolone sulfate; PROG, progesterone; E_2, 17 -estradiol; NP, Nonylphenol; OP, 4-octylphenol; DBP, di-n-butyl phthalate; DEP, di-2-ethylhexyl phthalate; DES, diethylstilbestrol - HEX, -hexosaminidase; PTX, pertussis toxin; PLC, phospholipase C; DPH, diphenhydramine; AS-ODN, antisense oligodeoxynucleotide; MS-ODN, missense oligodeoxynucleotide

Introduction

Steroids synthesized *de novo* in the central and peripheral nervous systems are called neurosteroids, and have a wide variety of physiological and pharmacological functions (Compagnone and Mellon 2000; Plassart-Schiess and Baulieu 2001). Although steroid hormones are known to have genomic actions through intracellular steroid hormone receptors (Beato and Klug 2000), there is also increasing evidence of rapid nongenomic steroid actions (Rupprecht and Holsboer 1999; Falkenstein et al. 2000). The latter nongenomic actions of neurosteroids include allosteric actions on ligand-gated channels such as $GABA_A$ (Majewska et al. 1986) and NMDA (Wu et al. 1991) receptors, and these actions influence neuronal excitability (Majewska et al. 1986). Recently, it has been found that progesterone binds to the oxytocin receptor, a member of the G protein-coupled receptor (GPCR) family, and suppresses oxytocin-induced responses (Grazzini et al. 1998). On the other hand, we have reported that some neurosteroids share pharmacological actions with a putative G_i-coupled sigma ( ) receptor in the brain through reconstitution experiments (Ueda et al. 2001). Thus, neurosteroids may have nongenomic mechanisms through GPCRs in addition to ligand-gated channels.

Recently, we reported that pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulfate (DHEAS), two representative neurosteroids, induce nociceptive flexor responses through two novel types of neurosteroid receptors, termed neuronal NS1/ -type and non-neuronal NS2-type (Ueda et al. 2001a). The NS2-type neurosteroid receptor probably exists on mast cells to induce histamine release, since the nociceptive responses and plasma extravasation after neurosteroid stimulation were completely blocked by diphenhydramine (DPH), a histamine antagonist (Ueda et al.

2001a ; Uchida et al. 2003). While studying the physiological and pathophysiological roles of this peripheral neurosteroid receptor, we found that its neurosteroid-induced actions were abolished by 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE), an endocrine-disrupting chemical (EDC) that possesses estrogenic actions (Uchida et al. 2003). Therefore, the peripheral neurosteroid receptor may be a target for some EDCs as well as nuclear steroid receptors. In this study, we demonstrate the involvement of the putative $G_{q/11}$-coupled neurosteroid receptor in the mast cell degranulation induced by neurosteroids and EDCs.

Methods

Materials

The following chemicals were obtained from Sigma (St. Louis, MO): pregnenolone sulfate (PREGS), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), progesterone (PROG), Progesterone 3(*O*-Carboxymethyl) oxime conjugated to bovine serum albumin (PROG-BSA) (steroids:BSA = 38:1), PROG-BSA-fluorescein isothiocyanate conjugate (PROG-BSA-FITC), 17 -estradiol (E_2), E_2-6-*O*-carboxy-methyl oxime-BSA (E_2-BSA) (steroids: BSA = 32:1), N-acetyl- -D-glucosaminide, oxytocin, quercetin and RU486. ICI-182,780 was purchased from WAKO (Osaka, Japan). Nonylphenol (NP), 4-octylphenol (OP), di-n-butyl phthalate (DBP), di-2-ethylhexyl phthalate (DEP), and diethylstilbestrol (DES) were purchased from KANTO KAGAKU (Tokyo, Japan). Xestospongin C (araguspongine E) was a gift from Prof. Kobayashi (Kobayashi et al. 1989). The antisense oligodeoxynucleotide (AS-ODN, 5'-ATGGACTCCAGAGT-3') for rat $G_{q/11}$ and its missense oligodeoxynucleotide (MS-ODN, 5'-AGTGACCTCAGGAT-3') were synthesized (Ueda and Inoue, 2000). The antisense oligodeoxynucleotide (AS-ODN, 5'-TCGGCAGCCATGATCTCT-3') for rat 25-DX and its missense oligodeoxynucleotide (MS-ODN, 5'-TCCGCTGCG ATCATGTCA-3') were synthesized. All ODNs were purchased from QIAGEN (Tokyo, Japan). $G_{q/11}$ was obtained from NEN Life Science Products, Boston, MA. Antiserum rabbit against 25-DX (IZA) was a gift from Prof. Okamoto (Raza et al., 2001). We calculated the concentration of BSA-bound steroids as reported previously (Ueda et al., 2001a).

Cell Culture

RBL-2H3 cells were maintained in minimum essential medium supplemented with 10% FBS, L-glutamine and penicillin/streptomycin. In most experiments, unless otherwise stated, RBL-2H3 cells were treated with 30 M quercetin for 48 h, as described previously (Senyshyn et al. 1998). Quercetin was prepared as a 60 mM stock solution in propylene glycol. For each experiment, the cells were incubated overnight in complete growth medium and then with pertussis toxin (PTX; 100 ng • ml^{-1}) for 12 h, or U-73122 (100 nM), U-73343 (100 nM), xestospongin C (1 M), EGTA (1 mM), wortmannin (1 M), RU-486 (1 M) or ICI-182,780 (1 M) for 10 min before addition of the test drugs. The AS-ODN or MS-ODN for $G_{q/11}$ (20 M), 25-DX (20 M) was pretreated every 8 h for 48 h before addition of the test drug for measurement of -HEX release. Western blot analysis was performed as reported previously (Ueda and Inoue 2000), using the antiserum against $G_{q/11}$ (1:1000 dilution) or 25-DX (1:1000 dilution).

Measurement of -Hexosaminidase Release

The experiment was performed according to previous reports (Hong-Geller and Cerione 2000; Zussman and Sagi-Eisenberg 2000). RBL-2H3 cells were seeded in 24-well plates, grown to confluency, incubated in growth medium containing 30 M quercetin, and then washed twice with HEPES-Tyrode's BSA buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 5.6 mM glucose, 12 mM $NaHCO_3$, 0.37 mM NaH_2PO_4 • $2H_2O$, 25 mM HEPES, 0.49 mM $MgCl_2$, 0.1% BSA, pH 7.4). The cells were then stimulated with various drugs for 10 min or indicated periods at 37°C, and 100 l aliquots of the supernatants were collected and incubated with 50 l of 10 mM N-

acetyl- -D-glucosaminide in 0.05 M citrate buffer (pH 4.5) for 1 h at 37°C. To determine the total amount of -hexosaminidase (-HEX), the cells were lysed with 0.1% Triton X-100. The absorbance at 405 nm was read in a microplate reader (Bio-Rad 550; Nippon Bio-Rad Laboratories, Osaka, Japan).

PROG-BSA-FITC Binding Assay

The experiment was performed according to previous reports (Benten et al. 1999; Nadal et al. 2000). RBL-2H3 cells (1.0 x 10^5 cells/well) cultured on polylysine-coated coverslips for 24 h were fixed with 4% (wt/vol) paraformaldehyde for 30 min, and then washed three times with phosphate-buffered saline (PBS). The cells were incubated with 1 M PROG-BSA-FITC at room temperature for 2 h, and then washed. FITC-labeled cells were analyzed using a confocal laser scanning microscope (FLUOVIEW; Olympus, Tokyo, Japan), after excitation of the FITC fluorescence by a 488 nm argon laser line.

Animals and Behavioral Studies

The algogenic-induced biting and licking (ABL) test was performed as described previously (Uchida et al., 2003). Male ddY mice weighing 20 to 22 g were used in all experiments. The experimental procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983). Test drugs were intraplantarly (i.pl.) administered into the hind paw. Mice were adapted to individual transparent plastic cages, which served as observation chambers, for 1 h prior to the i.pl. injection. Immediately after the injection, each mouse was replaced in the cage over a mirror and

behavioral testing was initiated. The total amount of time showing nocifensive responses such as biting and licking behaviors was measured for 10 min after the i.pl. injection. We first dissolved neurosteroids and EDCs in 100 % methanol to 20 mM and diluted to a concentration of 1 - 30 fmol/20 l (or 5 pM – 1.5 nM) in physiological saline immediately before use. As stock DHEAS is highly diluted for *in vivo* use, any significant effects of methanol would be avoided. Test agents were administered by i.pl. injection in a volume of 20 l. Mice were only used once.

Statistical Analysis

The results were expressed as the mean ± S.E. and analyzed by a one-way ANOVA with Scheffe's test. The criterion of significance was set at $*p<0.05$.

Results

Neurosteroids Induce Rapid Degranulation of RBL-2H3 Cells

Degranulation of mast cells was determined by measuring the release of granule-associated -HEX. Significant and maximum -HEX releases were observed as early as 3 and 10 min after the addition of DHEAS (1 M), respectively (Fig. 1A). Similar agonistic activity was also observed with 1 M DHEA, DHEAS or PREGS, whereas no changes were observed with 1 M PROG, E_2, PROG-BSA or E_2-BSA (Fig. 1B). DHEAS did not cause any remarkable changes in the cell morphology or cell survival activity, as evaluated by the trypan blue exclusion assay (data not shown).

PROG-BSA and E_2 Antagonize the Degranulation

DHEAS (1 M)-induced degranulation was markedly blocked by PROG, E_2, PROG-BSA or E_2-BSA (Fig. 1C). Similar antagonism was also observed for PREGS-induced release. As shown in Fig. 1D, the concentration-dependent release by DHEAS (10 nM - 10 M) was shifted to the right by 10 M PROG-BSA.

EDCs Antagonize DHEAS-induced -HEX Release

As shown in Fig. 1E, DHEAS (1 M)-induced -HEX release was significantly inhibited by various EDCs, including 1 M nonylphenol (NP), 4-octylphenol (OP), di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEP), but not by 1 M diethylstilbestrol (DES). However, no significant changes in the degranulation were observed with these EDCs (Fig. 1F).