Table 2
Body weight changes in male rats treated neonatally with BPA or E2

	Control[a]	BPA[a]					E2[a]
	0 ng[b] 8[c]	24 ng[b] 8[c]	120 ng[b] 8[c]	600 ng[b] 8[c]	3 μg[b] 8[c]	1 mg[b] 8[c]	10 μg[b] 8[c]
PND 0	6.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.9 ± 0.1	7.0 ± 0.1	6.6 ± 0.1	7.0 ± 0.1
10	26.1 ± 0.7	26.1 ± 0.4	26.1 ± 0.5	26.2 ± 0.8	26.2 ± 0.7	25.3 ± 2.0	26.1 ± 2.0
21	64 ± 1	61 ± 2	62 ± 2	63 ± 2	62 ± 2	62 ± 5	62 ± 5
42	234 ± 6	233 ± 5	229 ± 7	234 ± 8	240 ± 8	243 ± 7	222 ± 7
63	409 ± 16	409 ± 13	397 ± 15	403 ± 8	420 ± 18	429 ± 16	411 ± 13
84	529 ± 27	528 ± 21	507 ± 24	506 ± 22	541 ± 24	549 ± 21	542 ± 15
105	594 ± 33	594 ± 24	554 ± 27	564 ± 25	605 ± 29	611 ± 22	616 ± 22
126	637 ± 40	631 ± 25	587 ± 28	595 ± 26	649 ± 32	652 ± 26	660 ± 23
140	674 ± 44	662 ± 25	612 ± 29	622 ± 28	682 ± 33	681 ± 30	680 ± 22
150	699 ± 46	688 ± 26	630 ± 30	644 ± 29	704 ± 33	708 ± 31	710 ± 22

Each value shows mean ± S.E.
[a] Group.
[b] Dose.
[c] Number of males.

Table 3
Preputial separation in male rats treated neonatally with BPA or E2

	Control[a]	BPA[a]					E2[a]
	0 ng[b] 8[c]	24 ng[b] 8[c]	120 ng[b] 8[c]	600 ng[b] 8[c]	3 μg[b] 8[c]	1 mg[b] 8[c]	10 μg[b] 8[c]
Number of males in which preputial separation was completed	8	8	8	8	8	8	3
Number of males in which preputial separation was incompleted	0	0	0	0	0	0	5
Days when preputial separation was completed	45 ± 1.1	44 ± 0.7	44 ± 1.1	42 ± 0.6	44 ± 0.9	44 ± 0.3	590.9[d,**]

Each value shows mean ± S.E.
[a] Group.
[b] Dose.
[c] Number of males.
[d] Mean in three males.
** $P < 0.01$.

Table 4
Copulatory rate and fertilty rate in male rats treated neonatally with BPA or E2

	Control[a]	BPA[a]					E2[a]
	0 ng[b] 8[c]	24 ng[b] 8[c]	120 ng[b] 8[c]	600 ng[b] 8[c]	3 μg[b] 8[c]	1 mg[b] 8[c]	10 μg[b] 8[c]
Paring							
First paring	8	8	7	8	7	7	3
Second paring	–	–	1	–	1	1	0
Third paring	–	–	–	–	–	–	0
Fourth paring	–	–	–	–	–	–	0
Total	8	8	8	8	8	8	3
Copulatory rate (%)[d]	100.0	100.0	100.0	100.0	100.0	100.0	37.5[*]
Number of pregnant animals	8	8	8	8	8	7	2
Fertility rate (%)[e]	100.0	100.0	100.0	100.0	100.0	87.5	66.7

–: Not mated.
[a] Group.
[b] Dose.
[c] Number of males.
[d] (Number of females with successful copulation/number of females) × 100.
[e] (Number of pregnant females/number of females with successful copulation) × 100.
* $P < 0.05$.

Table 5
Observation of fetuses derived from male rats treated with neonatally BPA or E2

	Control[a]	BPA[a]					E2[a]
	0 ng[b] 8[c]	24 ng[b] 8[c]	120 ng[b] 8[c]	600 ng[b] 8[c]	3 μg[b] 8[c]	1 mg[b] 7[c]	10 μg[b] 2[c]
Number of copora lutea	16.1 ± 0.5	16.5 ± 0.6	17.0 ± 0.5	16.6 ± 0.6	16.5 ± 0.6	17.4 ± 1.6	16.0
Number of implantation sites	15.3 ± 0.6	14.3 ± 1.2	16.5 ± 0.4	15.8 ± 0.6	16.0 ± 0.5	16.0 ± 0.6	14.0
Implantation rate[d]	94.5 ± 2.2	84.7 ± 5.1	97.2 ± 1.1	94.8 ± 2.3	97.2 ± 2.0	91.9 ± 2.2	87.9
Post-implantation loss	0.8 ± 0.4	0.6 ± 0.2	2.5 ± 1.5	0.6 ± 0.2	0.9 ± 0.5	1.1 ± 0.6	0.0
Number of live fetuses	14.5 ± 0.4	13.6 ± 1.2	14.0 ± 1.5	15.1 ± 0.6	15.1 ± 0.7	14.9 ± 1.0	14.0

Each value shows mean ± S.E.
[a] Group.
[b] Dose.
[c] Number of dams.
[d] (Number of implantation sites/number of copora lutea) × 100.

Table 6
Sperm analysis in male rats treated neonatally with BPA or E2

	Control[a]	BPA[a]					E2[a]
	0 ng[b] 8[c]	24 ng[b] 8[c]	120 ng[b] 8[c]	600 ng[b] 8[c]	3 μg[b] 8[c]	1 mg[b] 8[c]	10 μg[b] 8[c]
Sperm motility							
Motile sperm rate (%)	79.7 ± 2.8	77.0 ± 1.1	79.4 ± 0.6	77.8 ± 2.2	79.9 ± 1.3	79.4 ± 1.5	80.0 ± 1.3
Progressive sperm rate (%)	41.3 ± 3.5	38.7 ± 1.9	40.1 ± 3.3	37.8 ± 3.3	40.9 ± 2.2	34.6 ± 1.9	36.8 ± 2.7
Sperm morphology							
Abnormal sperm rate (%)[d]	2.1 ± 0.5	0.7 ± 0.2*	1.0 ± 0.2	1.2 ± 0.3	0.9 ± 0.2*	2.2 ± 0.3	2.0 ± 0.6
Number of sperms in left cauda epididymis ($\times 10^6$)	380.2 ± 10.7	348.6 ± 22.2	342.9 ± 18.3	329.4 ± 23.8	357.5 ± 20.9	330.4 ± 15.1	232.2 ± 17**
Number of sperms/g of left cauda epididymis ($\times 10^6$)	1057 ± 49.5	981.4 ± 57.7	983.9 ± 47.8	945.8 ± 60.2	1017 ± 60.1	1012 ± 54.4	721.3 ± 42**

Each value shows mean ± S.E.
[a] Group.
[b] Dose.
[c] Number of males.
[d] (Number of abnormal sperms/number of sperms examined) × 100.
* $P<0.05$.
** $P<0.01$.

Results of the observation of fetuses in the BPA groups were not different from those in the control (Table 5). The fetal observation in the E2 group was similar to that of the control.

3.4. Sperm analysis

The motile sperm rate, progressive sperm rate, and number of sperm in left cauda epididymis in the BPA groups were not significantly different from the corresponding control values (Table 6). The significant decrease in the percent abnormal sperm in the 24 ng and 3 μg groups was considered spurious. The number of sperm per epididymis or per gram in the E2 group was significantly lower than in the control.

3.5. Organ weights

No changes in organ weights were noted in any of the BPA groups on PND 10, 35, or 150 (Table 7). In contrast, significant decreases in the organ weights were noted in the E2 group as follows: in the testis on PND 10, in the testis, seminal vesicle, ventral prostate, and penis on PND 35, and in the ventral prostate and penis on PND 150.

3.6. Serum testosterone levels

No changes in serum testosterone levels were noted in any of the BPA groups and the E2 group on PND 10, 35, or 150 (Table 8).

3.7. mRNA expression in the testis

No changes in mRNA expression in the testis were found in any of the BPA groups on PND 10 (Table 9), 35, or 150 (data not shown). In the E2 group, however, decreases in 3β-HSD and P450scc mRNA, and increases in ERβ, AR, and PR mRNA were evident on PND10 (Table 9), but not on PND 35 or 150 (data not shown).

Table 7
Organ weights in male rats treated neonatally with BPA or E2 PND10 Group

	Control[a]	BPA[a]					E2[a]
	0 ng[b] 8[c]	24 ng[b] 8[c]	120 ng[b] 8[c]	600 ng[b] 8[c]	3 μg[b] 8[c]	1 mg[b] 8[c]	10 μg[b] 8[c]
PND 10							
Testis (g/%)	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.3 ± 0.1**
PND 35							
Testis (g/%)	0.84 ± 0.02	0.86 ± 0.02	0.85 ± 0.02	0.82 ± 0.02	0.87 ± 0.01	0.81 ± 0.03	0.62 ± 0.04**
Epididymis (mg%)	98.0 ± 5.4	100.4 ± 3.7	102.0 ± 2.6	107.7 ± 7.6	99.5 ± 4.3	102.4 ± 2.7	91.8 ± 6.0
Seminal vesicle (mg%)	79.0 ± 4.3	94.2 ± 6.7	94.2 ± 3.0	88.9 ± 6.8	91.1 ± 5.1	81.6 ± 2.8	55.4 ± 4.1**
Ventral prostate (mg/%)	61.5 ± 3.7	63.9 ± 4.0	60.6 ± 3.9	58.7 ± 4.0	58.3 ± 2.8	53.9 ± 1.8	38.5 ± 3.8**
Penis (mg%)	40.3 ± 1.6	42.3 ± 1.6	42.4 ± 1.1	42.4 ± 1.0	39.7 ± 1.9	39.7 ± 0.6	25.9 ± 1.4**
PND 150							
Testis (g/%)	0.56 ± 0.01	0.55 ± 0.02	0.61 ± 0.04	0.59 ± 0.04	0.56 ± 0.02	0.52 ± 0.06	0.50 ± 0.03
Epididymis (mg%)	0.21 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.2 ± 0.01	0.19 ± 0.01	0.17 ± 0.01
Seminal vesicle (g%)	0.33 ± 0.03	0.32 ± 0.02	0.35 ± 0.02	0.37 ± 0.01	0.34 ± 0.02	0.31 ± 0.01	0.26 ± 0.02
Ventral prostate (mg/%)	110 ± 10	95 ± 8	115 ± 13	101 ± 6	97 ± 9	87 ± 10	66 ± 6**
Penis (mg/%)	27 ± 2	25 ± 2	28 ± 3	27 ± 1	25 ± 1	24 ± 1	19 ± 2**

Each value shows mean ± S.E.
[a] Group.
[b] Dose.
[c] Number of males.
** $P<0.01$.

Table 8
Serum testosterone levels in male rats treated neonatally with BPA or E2

	Control[a]	BPA[a]					E2[a]
	0 ng[b]	24 ng[b]	120 ng[b]	600 ng[b]	3 μg[b]	1 mg[b]	10 μg[b]
PND 10[c]	0.15 ± 0.08	0.18 ± 0.06	0.16 ± 0.05	0.22 ± 0.07	0.25 ± 0.14	0.13 ± 0.06	0.10 ± 0.04
PND 35[d]	0.20 ± 0.07	0.21 ± 0.08	0.12 ± 0.03	0.17 ± 0.05	0.27 ± 0.09	0.16 ± 0.06	0.38 ± 0.11
PND 150[d]	3.03 ± 0.72	1.97 ± 0.44	2.63 ± 0.44	2.37 ± 0.32	1.80 ± 0.29	2.07 ± 0.51	2.23 ± 0.31

Each value shows mean ± S.E.
[a] Group.
[b] Dose.
[c] Serum samples from two or three animals of the same group were put together and regarded as one test sample; a total of three test samples thus obtained from eight animals were subjected to measurement.
[d] Serum samples from eight animals per group, were individually subjected to measurement.

Table 9
mRNA relative expression[a] in the testis in male rats treated neonatally with BPA or E2 (PND 10)

	Control[b]	BPA[b]					E2[b]
	0 ng[c] 8[d]	24 ng[c] 8[d]	120 ng[c] 8[d]	600 ng[c] 8[d]	3 μg[c] 8[d]	1 mg[c] 8[d]	10 μg[c] 8[d]
3β-HSD	1.00 ± 0.09	1.01 ± 0.09	0.84 ± 0.09	0.85 ± 0.09	0.84 ± 0.07	0.84 ± 0.11	0.71 ± 0.08*
P450scc	1.00 ± 0.05	1.00 ± 0.08	0.92 ± 0.09	0.88 ± 0.13	1.09 ± 0.12	0.96 ± 0.08	0.31 ± 0.13**
ERβ	1.00 ± 0.09	1.03 ± 0.05	0.89 ± 0.11	0.92 ± 0.11	0.82 ± 0.14	0.77 ± 0.11	1.71 ± 0.27*
AR	1.00 ± 0.13	1.01 ± 0.15	0.79 ± 0.08	0.87 ± 0.09	0.74 ± 0.09	0.70 ± 0.13	1.70 ± 0.22*
PR	1.00 ± 0.15	1.32 ± 0.15	0.92 ± 0.13	1.12 ± 0.22	1.13 ± 0.25	1.34 ± 0.14	17.97 ± 5.83*

Each value shows mean ± S.E.
[a] Only for genes for which significant differences from the control group were seen on PND 10 in any group.
[b] Group.
[c] Dose.
[d] Number of males.
* $P<0.05$.
** $P<0.01$.

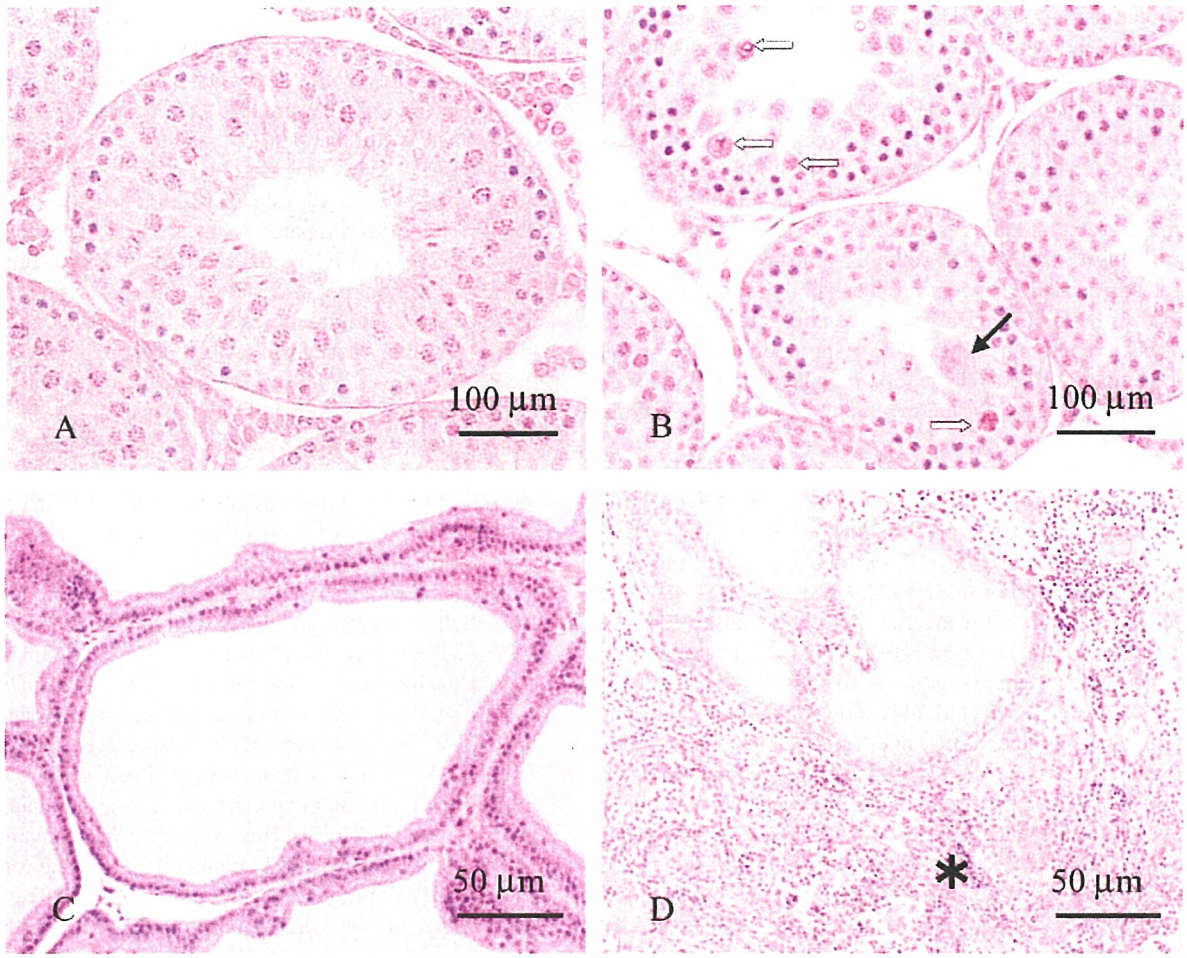

Fig. 1. Photomicrographs of the testicular tissue and on PND 35 from control group (A) and E2 group (B). The ventral prostate on PND 150 from control group (C) and E2 group (D). (A) Normal appearance in semiferous tubule; (B) degeneration of germinal epithelium with a multinucleated giant cell (black arrow) and apoptotic cells (white arrows) is found; (C) normal appearance in prostate; (D) chronic prostatitis with acute inflammation, which was mainly characterized by lymphocytic infiltration of the interstitium (asterisk). H.E. stain.

3.8. Histological changes in the testis and ventral prostate

Compared to the control group, no changes in the testis or ventral prostate were found in any of the BPA groups on PND 10, 35, or 150, or in the E2 group on PND 10. Degeneration of germinal epithelium with multinucleated giant cells was found in three males in the E2 group on PND 35 (Fig. 1B). Apoptotic cells were found in some seminiferous tubles in the same three males as above. Chronic prostatitis with acute inflammation (Fig. 1D), which was mainly characterized by lymphocytic infiltration of the interstitium, was found in one male of the E2 group on PND 150.

4. Discussion

The present data revealed that neonatal exspoure to BPA caused no adverse effects on males and estrogen-mediated response in the testis. In contrast, E2 given neonatally elicited incomplete preputial separation, lower copulatory rate, decrease in the reproductive organ weight and number of sperm, degeneration of germinal epithelium, and alteration of mRNA expression in the testis. In the males given E2, however, no changes were found in the body weight, observation of fetuses, sperm analysis, or serum testosterone levels.

The severe damage caused by E2 on reproductive functions has been reported in male rodents treated perinatally with DES [36,37]. Changes in the testis, that is, gene expression on PND 10, decrease in the testis weight on PNDs 10 and 35, and degeneration of germinal epithelium on PND 35, were temporary, and disappeared by PND 150. However, the effect on the number of sperm in the left cauda epididymis remained until PND 150. Also, the weights of the penis and prostate on PND 150 were affected by neonatal treatment with E2. Abnormalities in the prostate have also been reported in male rodents perinatally treated with DES [38]. Thus, potent estrogens, like E2, have temporary and permanent adverse effects on male reproductive functions and fetal development. Serum testosterone levels were normal at all ages examined in the E2 group despite the presence of effects of E2 on testosterone-dependent reproductive endpoints such as preputial separation, organ weights,

sperm counts, and expression of steroidogenic enzymes. This discrepancy may be ascribable to the decreased sensitivity of the seminiferous tubules and accessory organs to testosterone caused by estrogen given neonatally [39,40], since estrogen given neonatally is assumed to act directly on neonatal testes as well as on the hypothalamic mechanisms [30]. Occurrence of numerous multinucleated giant cells in the seminiferous tubules of the E2 group on PND 35 indicates the direct action of estrogen on the testis and/or on the hypothalamo-hypophyseal axis in neonates as has been suggested in neonatally estrogenized mice [39–41], although the effect of estrogen may be transient in rats as has been reported by others [42]. Reproductive abnormalities caused by neonatal estrogenization in male is known to depend on the dose and length of the treatment, and species of animals [30].

The doses of BPA per kg body weight were 2 μg (1–3.5 μg) in the 24 ng group, 11 μg (4.8–18 μg) in the 120 ng group, 56 μg (24–87 μg) in the 600 ng group, 277 μg (124–429 μg) in the 3 μg group, and 97 mg (43–152 mg/kg) in the 1 mg group. The dose of E2 per kg body weight was 0.9 mg (0.4–1.4 mg) in the 10 μg group. BPA even at the highest dose (97 mg/kg) has no effect on male reproductive functions or fetal development, being in agreement with Nagao's result [43] at a dose of 300 mg/kg. Atanassova et al. [44] showed that the testis weight was greater than the control values on PNDs 18 and 90–100 in Wistar rats treated neonatally with BPA at 0.5 mg daily from PNDs 1 to 11. In the present study, however, the testis weight of rats treated neonatally with BPA at 1 mg (97 mg/kg) daily from PNDs 0 to 9 was of the same order as that of the controls throughout the experimental period. In a 3-generation reproductive toxicity study of dietary BPA in rats [45], no adverse effect was observed at reproductive and postnatal stages with 750 ppm (50 mg/kg/day). Taking account of the results in the 1 mg group (97 mg/kg), the highest dose level in the present study, BPA may have no reproductive toxicity even at fairly high dose levels.

On the other hand, no effects of BPA at dose levels low enough to express in μg/kg, at which prostate weight increased [22] and daily sperm production reduced [21] in male mice treated prenatally, were noted in male rats treated neonatally with BPA at 2–277 μg/kg. Thus the results of the present study at low-dose levels were different from those obtained in previous studies [21,22]. It cannot be said, however, that the former was absolutely discrepant with the latter, since the experimental design was different in the dosing route, dose levels, dosing duration, and species. Pottenger et al. [46] reported that the relative bioavailability of BPA in rats was markedly lower following oral administration as compared to subcutaneous administration. Therefore, the subcutaneous dosages we employed have the possibility of causing higher bioavailability than the oral dosages vom Saal et al. [21] had employed. As described in this study, PR in the testis, an estrogen-responsible gene, was not up-regulated at any of the dosages used in the present study; it is safe to conclude that estrogen activity of BPA was not detected. The results of the present study are in agreement with those of Cagen et al. [26]. It seems highly probable, therefore, that the present findings verify a number of hypotheses on the low-dose effect of BPA on male rats.

The testis plays an important role in sex differentiation of the brain by testosterone secretion in the perinatal period. In the present study, some of the genes in steroidogenic enzymes were selected and determined as an indication of functions in the testis in addition to genes of sex steroid receptors, ERs, AR, and PR. Alterations of gene expression were noted in the E2 group only on PND 10. E2 caused high gene expression of PR about a 17-folds compared to the control. The up-regulation of PR mRNA by E2 demonstrates that ER is functional in the testis. Although it was shown in the rat hypothalamus that BPA increased PR immunoreactivity [47] and mRNA expression [48], BPA at any dose level evoked no up-regulation at least in the neonatal testis. P450scc, which metabolizes the cholesterol to the pregnenolone (3β-hydroxypregn-5-en-20-one), and 3β-HSD, which metabolizes the pregnenolone and/or its analog, both genes of steroidogenic enzymes, were down-regulated in the E2 group, but not in any of the BPA groups. In addition to the change in the steroidogenic enzyme, ERβ, and AR mRNA expression were affected by E2, but not by BPA. A transient increase in serum testosterone has been reported in F1 male rats from mothers treated prenatally with BPA at 25 and 250 μg/kg [49] or perinatally at 4 and 40 mg/kg [50], but in this study BPA caused no changes in serum testosterone levels at any dose level.

In a study of BPA using the same neonatal model as in the present study, some abnormalities have been detected in the reproductive organs of females treated neonatally with BPA at high dose levels (1 and/or 4 mg/female; almost the same doses as in the present study) [34]. However, since the neonatal treatment with BPA at any dose level had no effect on male reproductive functions, BPA produces hardly any injurious effect on reproduction in the males when exposed to BPA after birth.

In conclusion, neonatal BPA treatment causes no adverse effects both on male reproductive functions and on gene expression for the steroidogenic enzymes in the testis.

Acknowledgments

The authors greatly acknowledge Emeritus Professor Noboru Takasugi at Yokohama City University and Dr. Raphael Guzman at Department of Molecular Cell Biology, University of California, Berkeley, for their valuable advice and critical readings of this manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (A) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Health Science Research Grant from the Ministry of Health, Labor and Welfare, Japan.

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Global Gene Expression in Mouse Vaginae Exposed to Diethylstilbestrol at Different Ages

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Estrogens regulate proliferation and differentiation of cells in target organs such as the female reproductive tract. In mature mice, estrogens stimulate cell proliferation, whereas ovariectomy results in atrophy of the female reproductive tract. In contrast, perinatal exposure to estrogens, including diethylstilbestrol (DES), induces persistent, ovary-independent vaginal stratification and cervico-vaginal tumors later in life. These effects are due to altered cell fate following DES exposure during a critical developmental period. The detailed mechanisms underlying the reversible and irreversible cell proliferation in vaginae induced by DES at different ages has not been clarified. Therefore, we examined differences in gene expression pattern using DNA microarray analysis in mouse vaginae 6 hrs after a single injection of 2 μg DES per gram of body weight, and proliferation of vaginal epithelial and stromal cells 24 hrs after the injection at postnatal days (PNDs) 0, 5, 20, and 70. After DES stimulation, vaginal epithelial and stromal cells showed cell proliferation at PNDs 20 and 70, and at PNDs 0 and 5, respectively. DNA microarray analysis exhibited 54 DES-induced genes and 9 DES-repressed genes in vaginae at PND 0, whereas more than 200 DES-induced genes were found in vaginae at PNDs 5 and 20, and 350 genes at PND 70. Clustering analysis of DES-induced genes in the vaginae at different ages revealed that genes induced by DES at PND 5 were closer to the adult type than that of PND 0. Genes related to keratinocyte differentiation, such as Gadd45α, p21, 14–3–3 sigma, small proline-rich protein 2f (Sprr2f), and Krupple-like factor 4 (Klf4), were induced by DES. The number of DES-induced genes during the critical period, PND 0, was smaller than those found after the critical period. These results give insight toward understanding the molecular mechanisms underlying the critical period in mouse vaginae. Exp Biol Med 231:632–640, 2006

Key words: microarray; gene expression; diethylstilbestrol; vagina; mouse

Estrogens induce cell proliferation and differentiation, whereas estrogen depletion results in atrophy accompanied by apoptosis in adult female reproductive organs such as the uterus and vagina (1–3). Estrogen exposure during a critical period in early development induces persistent ovary-independent proliferation and keratinization in the vaginal epithelium during adulthood (4, 5). Diethylstilbestrol (DES), a synthetic estrogen used to prevent miscarriage during the 1940s to the early 1970s, induced vaginal clear cell carcinoma and uterine abnormalities in daughters of mothers exposed to DES during pregnancy (6). Similar abnormalities were reported in mice exposed to estrogens during a critical perinatal period (4, 5, 7). In female mice, various abnormalities such as polyovular follicles, oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization, vaginal

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by a Health Sciences Research grant from the Ministry of Health, Labour and Welfare; and by a research grant from the Ministry of Environment, Japan.

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Received January 12, 2006.
Accepted February 14, 2006.

1535-3702/06/2315-0632$15.00

adenosis, and cervico-vaginal carcinomas, were induced by perinatal exposure to estrogens including DES (4, 5, 7–13). Vaginal epithelial proliferation persists even after ovariectomy in adult mice exposed to a sufficient dose of DES during the early neonatal period (4, 5).

During the normal estrous cycle, vaginal epithelial cell proliferation and keratinization occur at the estrous stage (1), whereas keratin 1 (K1) and progesterone receptor expressions were induced at the proestrous stage (14, 15). DES exposure during a critical developmental period results in alteration of the response to estrogen in the vagina, leading to a set of subsequent abnormalities. Epithelial cells failed to undergo apoptosis even after ovariectomy, and persistent expression of various genes was observed in the persistently proliferated vagina (15–17). Reduced expression of estrogen receptor (ER) mRNA and persistent expression of c-fos and c-jun mRNAs were observed in the vaginae of mice exposed to DES at the neonatal stage, even after ovariectomy (15). Persistent phosphorylation of erbB receptors, including epidermal growth factor (EGF) receptor, and sustained expression of EGF-like growth factors were found in the vaginae of mice neonatally exposed to DES (16). Neonatal exposure to a fibroblast growth factor family member, keratinocyte growth factor (KGF), resulted in persistent vaginal epithelial stratification (18). The induction of EGF by estrogens may play important roles in the proliferation of epithelial cells in the uterus and vagina (18–21).

We used DNA microarray to analyze gene expression in neonatally DES-exposed mouse vaginae and observed persistent expression of interleukin-1 (IL-1), IL-1 receptor, insulin-like growth factor-I (IGF-I) mRNAs, and stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), as well as phosphorylation of downstream genes (17).

The critical periods for the induction of abnormalities by estrogenic chemicals during mouse development varies by organ (22). Analyses of the molecular mechanisms underlying the critical sensitive window in each organ is essential for understanding the etiology of the persistent changes induced in the reproductive tracts. Therefore, we examined global expression in vaginae of early genes elicited by DES treatment at different ages in order to understand the differences in estrogen-responsive genes during and after the critical period, and in adulthood.

Materials and Methods

Animals. C57BL/6J mice (CLEA, Tokyo, Japan) were used at postnatal days (PNDs) 0, 5, 20, and 70. Mice were maintained in a 12:12-hr light:dark cycle at 23–25°C. They were fed a commercial diet (CE-2; CLEA), and tap water was provided *ad libitum*. All experiments and animal husbandry protocols were approved by the animal care committee of the National Institutes of Natural Sciences.

Treatments. DES (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil. Unless otherwise stated, all materials were obtained from Wako Pure Chemical Industries, Osaka, Japan. The day of birth was designated as Day 0. For microarray experiments, mice at PND 0 (7–12 mice from three litters), PND 5 (7–12 mice from three litters), PND 20 (8 mice), and PND 70 (8 mice) were given a single subcutaneous (sc) injection of 2 μg DES per gram of body weight (bw) or oil vehicle alone. Mice at PND 70 were ovariectomized at 56 days of age. Vaginae from DES-exposed and control mice were collected for DNA microarray analysis and quantitative real time–polymerase chain reaction (QRT-PCR). In order to identify early genes induced by DES, tissues were dissected 6 hrs after the injection as described previously (23–25).

In addition, five mice each were given a single sc injection of 2 μg DES/gram bw or oil vehicle alone for the bromodeoxyuridine (BrdU) experiment.

DNA Microarray Analysis. Total RNA from vaginae was extracted using TRIZOL (Invitrogen, Tokyo, Japan) and purified using an RNeasy mini kit (Qiagen, Tokyo, Japan). Quality and quantity of total RNA were confirmed by the Agilent 2100 bioanalyzer (Agilent, Tokyo, Japan). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix for use on their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix, Inc.) containing approximately 12,500 genes, and the scanned data were analyzed with GeneChip software (Affymetrix, Inc.) and processed as described previously (23). To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, we independently repeated the same experiment at least twice. The expression data were analyzed with GeneSpring software (Agilent, Palo Alto, CA) as described previously (26).

For the clustering analysis, genes activated more than 2-fold by DES were selected, and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described (23–26). Gene expression change was estimated by the value of control PND 0 as one. Putative target genes were validated by QRT-PCR.

QRT-PCR. Total RNA was purified as described above. cDNA was synthesized from purified total RNA with Superscript II RT (–) (Invitrogen) with random primers at 42°C for 60 mins. PCR reactions were performed in the PE Prism 5700 sequence detector (PE Biosystems, Tokyo, Japan) using SYBR-Green PCR core reagents (PE Biosystems) in the presence of appropriate primers according to the manufacturer's instructions. PCR amplification was performed in triplicate under the following conditions: 2 mins at 50°C, 10 mins at 95°C, followed by a total of 40 cycles of 15 secs at 95°C and 1 min at 60°C.

Gene expression levels were normalized to the expression levels of ribosomal protein L8 mRNA

Table 1. Sequences for Primers Used for QRT-PCR

Genbank accession No.	Name	Forward primer	Reverse primer
U20344	Klf4	ACACAGGCGAGAAACCTTACCA	AATTTCCACCCACAGCCGT
AF058798	14-3-3 sigma	ACAAGGACAGCACCCTCATCA	ACAGCGTCAGGTTGTCTCTCAG
AJ005559	Sprr2a	TCCTGTAGTGTGCTATGAGCAATG	TTGCACAGGAGGGCATGTT
AJ005564	Sprr2f	TGAGGCTTCAGCAACAATGTCTT	TTGGTGGTGGACACACAGGA
AB003502	Gspt1[a]	CAAGTATGCATTGCGCGTTTA	CCCATCTGAGGGAAGTCCTTAA
AI121305	EST	TTATGTCCTCAGTCCGCAGCT	TAGTGTTGCAGGTCTGTGGTCC
AW048937	p21	TGAGACGCTTACAATCTGAGTGG	AACATGTATTGTGGCTCCCTCC
U00937	Gadd45α	GAAGAAGGAAGCTGCGAGAAAA	CCTGGCCATCCTAAATTAGCAGT
U67771	Ribosomal protein L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT

[a] Gspt1, G1 to S phase transcript 1.

(U67771), and gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands. The primers were chosen to amplify short PCR products of less than 100 base pairs and their sequences are listed in Table 1.

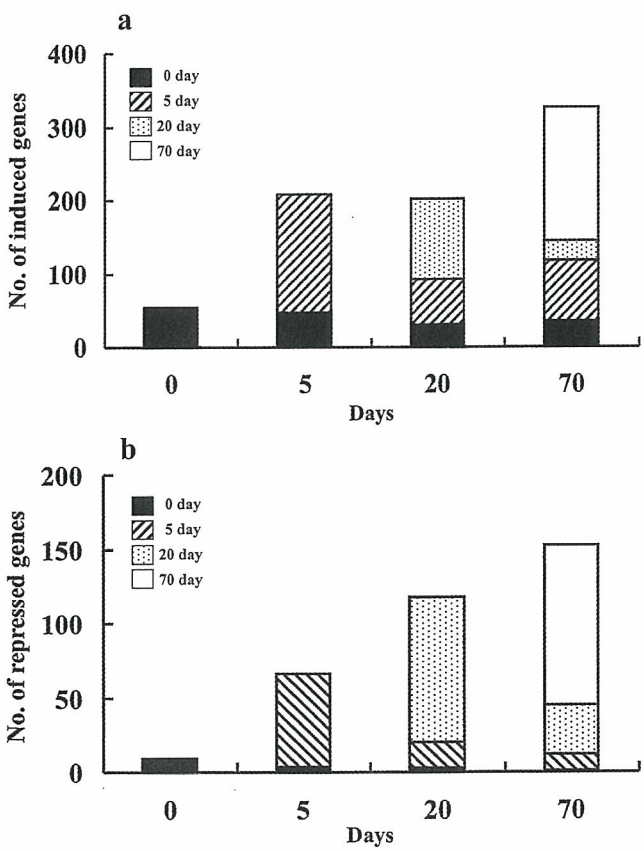

Figure 1. (a) The number of induced genes in vaginae 6 hrs after a single injection of 2 μg DES/g bw at PNDs 0, 5, 20, and 70. Mice at PND 70 were ovariectomized 2 weeks before. The number of DES-induced genes was small at PND 0, but it increased drastically at PND 5. (b) The number of repressed genes in vaginae 6 hrs after a single injection of 2 μg DES/g bw at PNDs 0, 5, 20, and 70. The numbers of DES-repressed genes increased linearly with age. Each pattern in the bar indicates genes commonly induced (a) or repressed (b) genes in respective ages. Days indicate ages given a single injection of DES.

Gene expression levels in DES-treated groups were normalized using control PND 0 as one. Parametric variables were analyzed by one-way analysis of variance (ANOVA) with post-hoc Student's *t* test or Welch's *t* test.

Immunostaining of BrdU. Five mice each given a single sc injection of 2 μg DES were killed 24 hrs after the injection. Two hours before dissection, 0.05 mg/g BrdU (Sigma, Tokyo, Japan) was injected intraperitoneally. Tissues fixed with neutral-buffered 10% formalin were embedded in paraffin. Sections cut at 8 μm were incubated with 0.3% H_2O_2 in methanol for 30 mins at room temperature (RT) to block endogenous peroxidase activity. They were washed with 0.5% Tween-20 in phosphate-buffered saline (PBS), incubated with 2 *N* HCl in water for 20 mins at RT. They were washed with 0.5% Tween-20 in PBS, incubated with 1% bovine serum (BSA) for 20 mins at RT, and with anti-BrdU antibody (Roche, Mannheim, Germany) at a dilution of 1:15 in 1% BSA at 4°C overnight. Washing with 0.5% Tween-20 in PBS, sections were incubated with mixture of 3,3′-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. Counterstain was conducted with methyl green. The number of BrdU-positive cells in 300 epithelial cells in the middle part of vagina and that in 500 stromal cells were recorded. Proliferation rate (%) was estimated as a percentage of BrdU-positive cells in epithelial cells and stromal cells, separately.

Results

Gene Expression in the Vaginae of Mice Treated with DES at Different Ages. The number of detected genes in the mouse vagina from oil-injected controls was not very different among animals examined at different ages: newborn (PND 0) = 4988 genes, PND 5 = 4937 genes, PND 20 = 4881 genes, and PND 70 = 4903 genes. We selected genes showing at least a 2-fold change in expression in response to DES treatment for further analysis (listed at http://www.nibb.ac.jp/bioenv1/suzuki/). The number of genes induced or repressed by DES was modest at PND 0, but showed a sharp increase with age (Fig. 1). DES exposure induced 54, 208, 202, and 326 genes and repressed 9, 66, 117, and 152 genes in vaginae at PNDs

Table 2. Induced or Repressed Genes in Vaginae 6 Hrs After a Single Injection of DES at Only PND 0

Genbank accession No.	Fold change	Name
AV170770	2.0	EST
AI837116	2.4	Solute carrier family 41, member 1
AI851565	2.5	RIKEN cDNA 1500034J01 gene
U58887	3.3	SH3-domain GRB2-like 3
D50646	0.4	Stromal cell–derived factor 2
AI425990	0.4	RIKEN cDNA C530046L02 gene
AI646638	0.5	Frequently rearranged in advanced T-cell lymphomas 2
M12347	0.5	Actin, alpha 1, skeletal muscle
AI841689	0.5	Chemokine-like factor superfamily 3

0, 5, 20, and 70, respectively (Fig. 1). The number of genes (208) induced by DES at PND 5 was similar to that of PND 20 (202). Ninety-two of 208 (44%) genes induced by DES at PND 5 were also induced at PND 20 by DES. Fifty-eight (28%) PND 5–specific genes were induced by DES (Fig. 1). Four genes (including two enhanced sequence tags [ESTs]) were specific to PND 0 (Table 2 and Fig. 1).

The total number of DES-regulated genes was 781. Some genes showing drastic expression change by DES were selected for further study (Table 3). Many of these genes showed upregulation by a single injection of DES from PND 0 to PND 70. Twenty-five genes, including MAD2, G1 to phase transition 1, c-fos, early growth response 1 (Egr-1), and Kruppel-like factor 4 (Klf4) were induced by DES exposure at all ages examined. MAD2 is an estrogen-responsive gene and assembles the mitotic spindle at the G2/M checkpoint (27). c-fos and Egr-1 were reported to be estrogen-responsive genes in the mouse uterus or mammary gland (or both) (15, 23, 28). Klf4, an inhibitor of the G1/S phase, plays a role in keratinocyte differentiation (29, 30), and is identified as an estrogen-responsive gene in the present study (Table 3).

The number of DES-repressed genes showed an age-dependent increase (Fig. 1). Twenty of 66 (30%) DES-repressed genes at PND 5 were also found at PND 20. Thirty-nine of 117 (33%) DES-repressed genes at PND 20 were also found at PND 70, although the number of age-specific DES-repressed genes was 5 (56%), 39 (59%), 64 (55%), and 107 (70%) at PNDs 0, 5, 20, and 70, respectively (Table 2 and Fig. 1). One of the common DES-repressed genes at all ages was flavin-containing monooxygenase 1 (Fmo 1; Table 3), which regulates metabolism of chemicals (31).

Clustering Patterns and Category of DES-Regulated Genes in the Vaginae of Mice at Different Ages. The 729 genes showing more than a 2-fold change following a single injection of DES at PNDs 0, 5, 20, and 70 were used for clustering analysis. These genes can be grouped as PND 0, PND 5, and PND 70 in controls, and as PND 0 and PND 5–70 in DES-exposed vaginae (Fig. 2) because the clustering patterns of genes in DES-exposed vaginae at PND 5 was more similar to those of PND 20 and PND 70 than that of PND 0 (Fig. 2).

These genes could be categorized into several groups. Genes involved in cell proliferation (15%) and protein modification (17%) were found in the DES-induced genes at PND 0 (Table 4). DES-repressed genes at PND 0 included those involved in cell tissue structure (11%), defense response (11%), transcription (11%), and transport (22%) compared with other groups. DES-regulated genes categorized in organogenesis were repressed by DES at PNDs 5, 20, and 70.

Confirmation of Gene Expression by QRT-PCR. Expression of several genes showing upregulation by DES in mouse vaginae at different ages was confirmed using QRT-PCR. The fold change in gene expression was estimated based on the value of each gene in control PND 0 as one (Fig. 3). Expressions of 14–3–3 sigma , Klf4, Sprr2f, EST (AI121305), and Gadd45α, which promotes the G1 phase and acts at the G2/M checkpoint (32, 33), were increased with age in control mice. Expression of p21 and G1 to phase transition 1 mRNAs were increased at PND 5 in control mice, whereas expression of Sprr2a was decreased at PND 5 in control mice. Expression of these genes, except for Klf4 and EST (AI121305), were induced by DES at PNDs 5, 20, and 70, but not at PND 0. Expression of Klf4 and EST (AI121305) was upregulated by DES at PND 0.

Immunostaining of BrdU. In oil-treated controls, ratios of BrdU-positive cells were not different among postnatal ages either in epithelial cells or in stromal cells. BrdU-positive cells in the vaginal epithelium were increased at PNDs 5, 20, and 70 in DES-treated vaginae as compared with the oil controls. In contrast, a higher number of BrdU-positive cells were found in the stroma in PND 0 and PND 5 mice (Fig. 4).

Discussion

Estrogen, androgen, and KGF exposure for 5 days from the day of birth induces persistent vaginal epithelial stratification in mice (4, 7, 11, 16–18). The persistent vaginal epithelial stratification with superficial keratinization induced by perinatal estrogen exposure was reported to be accompanied by persistent expression of several growth

Table 3. Induced or Repressed Genes in Vaginae 6 Hrs After a Single Injection of DES at PNDs 0, 5, 20, and 70 Using DNA Microarray and QRT-PCR[a]

Genbank accession No.	Gene[b]	Age (days) 0	5	20	70
Induced genes		Ratio by microarray (by QRT-PCR)			
Cell proliferation					
AB003502	G1 to S phase transition 1 (Gspt1)	2.2 (1.4)	2.8 (1.6)	2.1 (2.4)	3.3 (1.2)
V00727	c-fos	4.0	14.5	6.4	9.4
U83902	MAD2	2.1	2.7	3.7	13.8
X59846	Gas6	4.0	3.9	2.1	3.7
AF058798	14-3-3 sigma	−(1.1)	3.4 (3.6)	3.4 (5.3)	2.7 (5.1)
AW048937	p21	−(1.5)	2.4 (1.6)	3.2 (3.9)	3.7 (2.5)
U00937	GADD45α	−(1.8)	6.0 (3.9)	4.5 (4.1)	4.5 (3.6)
Protein modification					
AB013848	Peptidyl arginine deiminase type I	4.1	6.8	3.3	4.5
L02526	Map2k1	2.1	2.1	2.2	2.0
X04591	Creatine kinase, brain	2.1	2.5	2.1	4.3
X59274	Protein kinase C, beta	2.6	2.7	2.1	2.2
Transcription					
M28845	Early growth response 1 (Egr-1)	2.8	4.7	2.9	3.5
U20344	Kruppel-like factor 4 (Klf4)	3.8 (3.5)	4.2 (5.0)	2.1 (4.2)	2.7 (2.0)
Signal cascade					
M63801	Gap junction membrane channel protein alpha 1	2.4	3.5	2.1	2.1
AI596360	RIKEN cDNA 4930422J18 gene	3.1	7.2	2.6	2.8
Unknown					
X67644	Immediate early response 3	2.7	5.3	3.7	2.7
AI121305	RIKEN cDNA 1600029D21 gene	3.8 (2.4)	15.1(14.9)	3.7 (4.3)	3.6 (2.4)
Cell structure					
K02108	K 2, basic, gene 6a	—	6.3	3.0	5.2
AB012042	K 2, basic, gene 6g	—	2.9	7.7	4.1
M36120	K 1, acidic, gene 19	—	—	—	2.6
AJ005559	Sprr2a	−(0.4)	2.2 (4.5)	−(1.3)	4.9 (4.0)
AJ005560	Sprr2b	—	—	—	4.4
AJ005564	Sprr2f	−(1.7)	15.0(14.2)	4.0 (8.0)	7.5 (3.9)
Repressed genes					
Transport					
D16215	Flavin-containing monooxygenase 1 (Fmo1)	0.5	0.3	0.3	0.5

[a] Gene expression change was calculated based on the value of PND 0 as one. QRT-PCR data appear in parentheses; — indicates no change.
[b] Gas6, growth arrest–specific 6; Map2k1, mitogen-activated protein kinase kinase 1; p21, cyclin-inhibitor 21; GADD45α, growth and DNA damage 45α; MAD2, mitotic arrest–deficient; Sprr, small proline-rich protein; K, keratin complex.

factors (16–19, 34, 35). However, the precise mechanism of estrogen effects on the vaginal epithelial proliferation at different ages has not been clearly demonstrated, although several studies demonstrated that neonatal DES exposure induced persistent expression of EGF and EGF-like growth factors (35, 36) and phosphorylation of ERα, EGF receptor, and erbB in mouse vaginae (16, 17).

In the present study, global gene expression in vaginae was analyzed at different ages using DNA microarray analysis. We demonstrated age differences in vaginal responses to estrogen in the induction of gene expression from PND 0 to PND 70. ER staining was found in vaginal stromal cells in CD-1 mice at PND 0 (37), and in both vaginal epithelial and stromal cells, but in only stromal cells in uterine epithelial cells in C57BL/Tw mice at PND 0 (38). Epithelial expression of ER in the reproductive tracts occurs later in C57BL/6 mice than in CD-1 mice (39). Developmental effects of estrogens, including DES on neonatal mouse vaginae, are mediated through stromal ER (40). In the present study, we used C57BL/6 mice. Thus, neonatal mouse vaginae seem to be responsive to estrogen at PND 0. However, in the present study, the number of DES-induced genes in vaginae at PND 0 was smaller compared with those in PNDs 5, 20, and 70 mice. Moreover, vaginal epithelia in mice at PNDs 5, 20, and 70 were proliferated by DES, but not at PND 0 in the present study (Fig. 4). The PND 0 mouse vagina is still under development even without estrogenic stimulation (4, 41). Thus, in terms of gene expression, the vagina at PND 0 is less sensitive to estrogenic stimulation than it is at later stages. It has been shown that the response to DES is different between the Müllerian and urogenital sinus vaginal epithelium; proliferation in the vaginal fornix (Müllerian vagina) is inhibited by neonatal DES and the cell morphology is altered (7). Further

Table 4. Functional Categories of DES-Responsive Genes in Mouse Vaginae Selected in the Present Study[a]

	Age (in days)							
	0		5		20		70	
Category	Induced	Repressed	Induced	Repressed	Induced	Repressed	Induced	Repressed
Biosynthesis	6	0	11	3	13	5	11	6
Cell proliferation	15	0	10	10	10	8	9	6
Cell tissue structure	2	11	9	10	9	5	9	3
Defense response	7	11	5	6	3	4	4	6
Metabolism	7	0	7	3	5	8	6	5
Mitochondrion	0	0	0	0	0	0	1	1
Organogenesis	0	0	0	4	0	3	1	3
Protein modification	17	0	12	7	12	7	9	5
Ribosome	2	0	1	1	0	1	1	0
Signal transduction	7	0	6	6	6	6	6	7
Transcription	7	11	11	10	10	14	12	16
Transport	7	22	7	9	13	13	11	7
Unclassified	23	45	21	31	19	26	20	35
Total (%)	100	100	100	100	100	100	100	100

[a] Total number of clustered genes in each category was 100%. Induced, % of categorized genes in DES-induced gene at each age; Repressed, % of categorized genes in DES-repressed gene at each age.

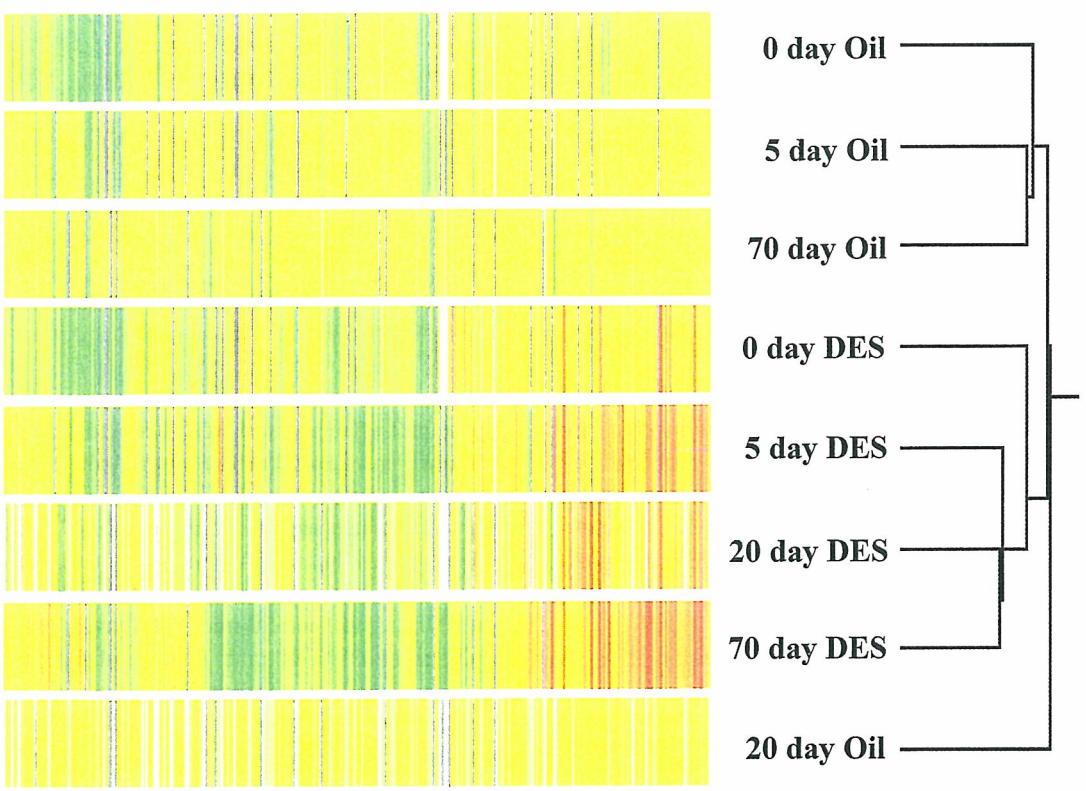

Figure 2. Clustering analysis of DES-responsive genes in mouse vaginae selected in the present study. Mice at PNDs 0, 5, 20, and 70 were stimulated by a single injection of DES. Each color bar indicates the expression level of one gene: red, induction; green, repression; yellow, average expression in eight groups; gray, not detected. Genes showing more than a 2-fold change in expression 6 hrs after a single injection of 2 μg DES/g bw at all ages were used for clustering analysis. Seven-hundred seventy-nine genes were selected. Control mice exhibited separated trees between the neonatal period (PNDs 0 and 5) and adulthood (PNDs 20 and 70). Branching of clustered genes in DES-exposed vaginae at PND 5 was closer to that of PND 0.

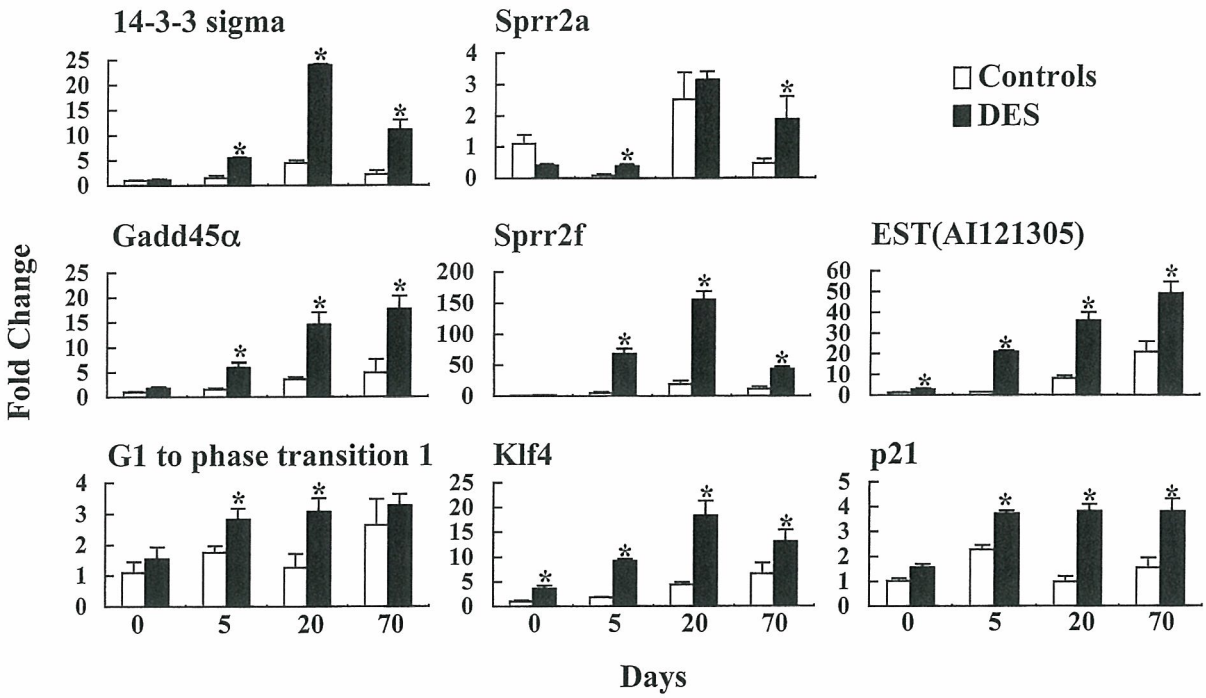

Figure 3. Changes in gene expression of cell cycle and keratinocyte differentiation regulators were confirmed by QRT-PCR. Fold change of gene expression by QRT-PCR was estimated based on the value of each gene at PND 0 as one. Results are the mean and SEM of three experiments. Each experiment was performed in triplicate. $P < 0.05$ vs. age-matched controls.

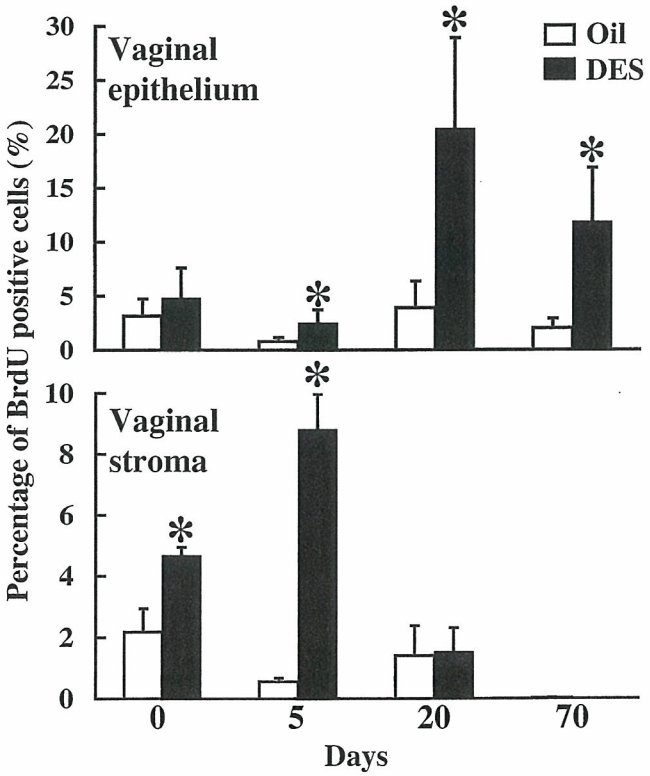

Figure 4. Percentage of BrdU-positive cells in vaginal epithelial cells and stromal cells at different ages. Mean and SEM. Note reverse of vaginal cell proliferation in epithelium and stroma after PND 5. *$P <$ 0.05 vs. age-matched controls.

studies examining differences in gene expression in Müllerian and urogenital sinus vagina in response to DES are essential.

Clustering analysis of estrogen-responsive genes in DES-exposed mouse vaginae showed that they could be broadly categorized into two types; a neonate type (PND 0) and an adult type (PNDs 5, 20, and 70). Vaginal stromal cells showed proliferative response to DES only at PNDs 0 and 5, but not at PNDs 20 and 70. In contrast, vaginal epithelial cells showed proliferative response to DES at PND 20 and PND 70 in the present study. The critical window for induction of estrogen-independent persistent changes in vaginae is within 3–5 days after birth (22). The underlying mechanism of the differences in responsiveness between vaginal epithelial cells and stromal cells at different ages needs to be analyzed in the near future to understand the molecular basis of the critical window. In mouse vaginae, proliferative response to DES in epithelial cells and stromal cells were reversed after PND 5, which may indicate the critical window of the mouse vagina.

Estrogens induce expression of genes related to cell cycle regulators, chromatin remodeling, IGF-I signaling, apoptosis, and keratin expression in mouse uteri (23–25, 42). Increased expression of mRNAs of cell cycle regulators were reported after 17β-estradiol treatment in the uteri of adult ovariectomized mice (42). In the present study, these cell cycle regulators except for cyclin G1 and E1, were induced in adult vaginae; thus vaginae responded to estrogen similar to uteri at the gene expression level.

From PND 5 onward, DES induced the following cell regulatory genes: p21, which delays S phase progression (43); Gadd45α, which acts at the G2/M checkpoint; and 14–3–3 sigma, which inhibits activation of cyclin B (32, 43, 44). Thus, induction of these cell cycle regulators at the mitotic phase checkpoint 6 hrs after DES stimulation may play a role in DNA synthesis required for vaginal cell proliferation.

Induction of keratinocyte differentiation regulators, such as Sprr1a, Sprr2a, and keratin complexes, was reported in estrogen-exposed uteri (45). Sprr family genes are expressed in all squamous cells, such as epithelial cells of skin, vagina, and digestive tract (46, 47). Sprr2a, Sprr2b, and Sprr2f are expressed in uteri and vaginae (48). Sprr2f is expressed most intensely in uteri and vaginae (48). Sprr2f and Keratin complex 2 (K2) mRNAs were elevated in vaginae 6 hrs after DES exposure from PND 5 to PND 70. Genes related to epithelial cell differentiation responded to DES earlier than genes related to proliferation at PND 5. The increase in expression of Sprr2f and K2 genes at PND 20 and PND 70 is probably related to proliferation of vaginal epithelial cells. Sprr2a and Sprr2b may be correlated with keratinization in vaginal epithelial cells.

Klf4 is a transcription factor of Sprr2 (47, 49) and plays a role in keratinocyte differentiation (30). DES induced Klf4 expression in vaginae even at PND 0 in the present study. The inductions of Klf4 and Sprr2a expressions in DES-exposed uteri at PND 5 were reported previously (45). In vaginae, Klf4 is an early estrogen-responsive gene and a candidate for persistent vaginal stratification by perinatal DES exposure.

14–3–3 Sigma also regulates the cell cycle by inhibiting G2/M progression-dependent p53 (44) and is induced in squamous cell carcinoma of the urinary bladder (50). Expression of 14–3–3 sigma was found in DES-stimulated mouse vaginae at PND 5 in the present study and also in neonatally DES-exposed vaginae (17), suggesting that this gene will be a candidate for further study in the persistent vaginal stratification and keratinization induced by perinatal estrogen exposure.

In conclusion, vaginal epithelial cells and stromal cells showed proliferation after a single injection of DES at PNDs 20 and 70, and at PNDs 0 and 5, respectively. The number of genes induced 6 hrs after DES exposure in mouse vaginae at PND 0 was lower than those induced at PNDs 5, 20, and 70. The DES-induced gene expression pattern in vaginae at PND 5 was closer to the adult type. Several cell cycle regulators such as Gadd45α, G1 to S phase transition 1, and p21; and keratinocyte differentiation factors, 14–3–3 sigma and Sprr2f, were induced by DES in vaginae from PND 5 to adult. Thus, microarray analysis revealed that the gene expression pattern in vaginae during the critical period was different from that after the critical period. Further studies are essential to examine the time course of gene expression to discover late genes induced in mouse vaginae by DES exposure at different ages.

We are grateful to Dr. Raphael Guzman, Department of Molecular Cell Biology, University of California at Berkeley and Cancer Research Laboratory, for his critical reading of this manuscript.

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0888-8809/06/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 20(9):2141–2155

doi: 10.1210/me.2005-0367

Endocrine-Disrupting Organotin Compounds Are Potent Inducers of Adipogenesis in Vertebrates

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Dietary and xenobiotic compounds can disrupt endocrine signaling, particularly of steroid receptors and sexual differentiation. Evidence is also mounting that implicates environmental agents in the growing epidemic of obesity. Despite a long-standing interest in such compounds, their identity has remained elusive. Here we show that the persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces the differentiation of adipocytes *in vitro* and increases adipose mass *in vivo*. TBT is a dual, nanomolar affinity ligand for both the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor γ (PPARγ). TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways *in vivo*. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased epididymal adipose mass in adults. In the amphibian *Xenopus laevis*, ectopic adipocytes form in and around gonadal tissues after organotin, RXR, or PPARγ ligand exposure. TBT represents, to our knowledge, the first example of an environmental endocrine disrupter that promotes adipogenesis through RXR and PPARγ activation. Developmental or chronic lifetime exposure to organotins may therefore act as a chemical stressor for obesity and related disorders. (*Molecular Endocrinology* 20: 2141–2155, 2006)

ORGANOTINS ARE A diverse group of widely distributed environmental pollutants. Tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), have pleiotropic adverse effects on both invertebrate and vertebrate endocrine systems. Organotins were first used in the 1960s as antifouling agents in marine shipping paints, although such use has been restricted in recent years. Organotins persist as prevalent contaminants in dietary sources, such as fish and shellfish, and through pesticide use on high-value food crops (1, 2). Additional human exposure to organotins may occur through their use as antifungal agents in wood treatments, industrial water systems, and textiles. Mono- and diorganotins are prevalently used as stabilizers in the manufacture of polyolefin plastics (polyvinyl chloride), which introduces the potential for transfer by contact with drinking water and foods.

Exposure to organotins such as TBT and TPTO results in imposex, the abnormal induction of male sex characteristics in female gastropod mollusks (3, 4). Bioaccumulation of organotins decreases aromatase activity leading to a rise in testosterone levels that promotes development of male characteristics (5). Imposex results in impaired reproductive fitness or sterility in the affected animals and is one of the clearest examples of environmental endocrine disruption. TBT exposure also leads to masculinization of at least two fish species (6, 7), but TBT is only reported to have modest adverse effects on mammalian male and female reproductive tracts and does not alter sex ratios (8, 9). Instead, hepatic-, neuro-, and immunotoxicity appear to be the predominant effects of organotin exposure (10). Hence, the current mechanistic understanding of the endocrine-disrupting potential of organotins is based on their direct actions on the levels or activity of key steroid-regulatory enzymes such as aromatase and more general toxicity mediated via damage to mitochondrial functions and subsequent cellular stress responses (11–15).

However, it remains an open question whether *in vivo* organotins act primarily as protein and enzyme

First Published Online April 13, 2006

Abbreviations: Acac, Acetyl-coenzyme A carboxylase; b.w., body weight; C/EBP, CCAAT/enhancer binding protein; 9-*cis* RA, 9-cis retinoic acid; DMSO, dimethylsulfoxide; F, forward; Fatp, fatty acid transport protein; LBD, ligand-binding domain; LXR, liver X receptor; MDIT, 3-isobutyl-1-methylxanthine, dexamethasone, insulin and T_3 adipocyte differentiation mix; PPAR, peroxisome proliferator-activated receptor; R, reverse; RAR, retinoic acid receptor; RXR, retinoid X receptor; Srebf1, sterol-regulatory element binding factor 1; TBT, tributyltin chloride; TPTO, triphenyltin oxide; TTNPB, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid; VDR, vitamin D receptor.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

inhibitors, or rather mediate their endocrine-disrupting effects at the transcriptional level. Recent work has shown that aromatase mRNA levels can be down-regulated in human ovarian granulosa cells by treatment with organotins or ligands for the nuclear hormone receptors, retinoid X receptor (RXRs) or peroxisome proliferator-activated receptor γ (PPARγ) (16–18). Furthermore, Nishikawa *et al.* (19) have demonstrated that the gastropod *Thais clavigara* RXR homolog is responsive to 9-cis-retinoic acid (9-*cis*-RA) and TBT, and 9-*cis* RA can also induce imposex, suggesting a conserved transcriptional mechanism for TBT action across phyla. These ligand-dependent transcription factors belong to the nuclear hormone receptor superfamily—a group of approximately 150 members (48 human genes) that includes the estrogen receptor, androgen receptor, glucocorticoid receptor, thyroid hormone receptor, vitamin D receptor (VDR), retinoic acid receptors (RARs and RXRs), PPARs, and numerous orphan receptors. We were therefore intrigued by the similar effects of TBT and RXR/PPARγ ligands on mammalian aromatase mRNA expression and hypothesized that TBT might be exerting some of its biological effects via transcriptional regulation of gene expression through activation of one or more nuclear hormone receptors.

Our results show that organotins such as TBT are indeed potent and efficacious agonistic ligands of the vertebrate nuclear receptors, retinoid X receptors (RXRs) and PPARγ. The physiological consequences of receptor activation predict that permissive RXR heterodimer target genes and downstream signaling cascades are sensitive to organotin misregulation. Consistent with this prediction we observe that organotins phenocopy the effects of RXR and PPARγ ligands using *in vitro* and *in vivo* models of adipogenesis. Therefore, TBT and related organotin compounds are the first of a potentially new class of environmental endocrine disrupters that targets adipogenesis by modulating the activity of key regulatory transcription factors in the adipogenic pathway, RXRα and PPARγ. The existence of such xenobiotic compounds was previously hypothesized (20, 21). Our results suggest that developmental exposure to TBT and its congeners that activate RXR/PPARγ might be expected to increase the incidence of obesity in exposed individuals and that chronic lifetime exposure could act as a potential chemical stressor for obesity and obesity-related disorders.

RESULTS

Organotins Are Agonists of Vertebrate RXR and RXR-Permissive Heterodimers

Many known or suspected environmental endocrine-disrupting chemicals mimic natural lipophilic hormones that act through members of the superfamily of nuclear receptor transcription factors (22, 23). In a screen of high-priority endocrine-disrupting chemicals against a bank of vertebrate nuclear receptor ligand-binding domains (LBDs), we observed that organotins, specifically tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPTO), could fully activate an RXRα LBD construct (GAL4-RXRα) in transient transfection assays. Both TBT and TPTO were as potent (EC_{50} ~3–10 nM) as 9-*cis* retinoic acid, an endogenous RXR ligand and approximately 2- to 5-fold less potent than the synthetic RXR-specific ligands LG100268 (EC_{50} ~ 2 nM) or AGN195203 (EC_{50} ~ 0.5 nM) (Fig. 1A and see Table 2). Maximal activation for TBT reached the same levels as LG100268 or AGN195203.

We next tested whether activation by TBT was unique to RXRα only, restricted to RXR heterodimer complexes, or a general nuclear receptor transcriptional response (Fig. 1, B–D, and Table 1). TBT activated RXRα and RXRγ from the amphibian *Xenopus laevis* in addition to human RXRs (Table 1). Our results are consistent with recent findings by Nishikawa *et al.* (19, 24) that organotins promote activation of all three human RXR subtypes in a yeast two-hybrid screen. We also observed significant activation of receptors typically considered to be permissive heterodimeric partners of RXR including human PPARγ (Fig. 1B, ~30% maximal activation of 10 μM troglitazone, but note that activation is compromised by cellular toxicity above 100 nM), PPARδ, liver X receptor (LXR), and the orphan receptor NURR1. In contrast, typical nonpermissive partners such as RARs, thyroid hormone receptor, and VDR failed to show activation by organotins (Fig. 1C and Table 1). Murine PPARα was also not activated by TBT although it was fully activated by its specific synthetic agonist WY-14643 (Fig. 1D). The steroid and xenobiotic receptor was likewise unresponsive. The orphan receptor NURR1, which has no discernable ligand pocket and is believed to be ligand independent (25), was nevertheless activated 7- to 10-fold at 100 nM TBT. Similarly, other RXR-specific ligands, *e.g.* LG100268, activated NURR1 to the same degree, suggesting that this response occurred through NURR1's heterodimeric partner RXR as has been previously described (25, 26). Like other RXR-specific ligands, tributyltin was also able to promote the ligand-dependent recruitment of nuclear receptor cofactors such as receptor-associated coactivator 3 (ACTR), steroid receptor coactivator-1, and PPAR-binding protein in mammalian two-hybrid interaction assays (data not shown). We infer from these results that nuclear receptor activation by TBT activation is specific to a small subset of receptors and not a consequence of a general effect on the cellular transcriptional machinery.

We next investigated the relationship between the structure of the tin compounds and RXR activation by testing the response of GAL4-RXRα to mono-, di-, tri-, and tetra-substituted butyltin, branched side chains, variations in the alkyl chain length, and changes in the halide component (Fig. 1A and Table 2). Overall, trialkyltin compounds were the most effective with nano-

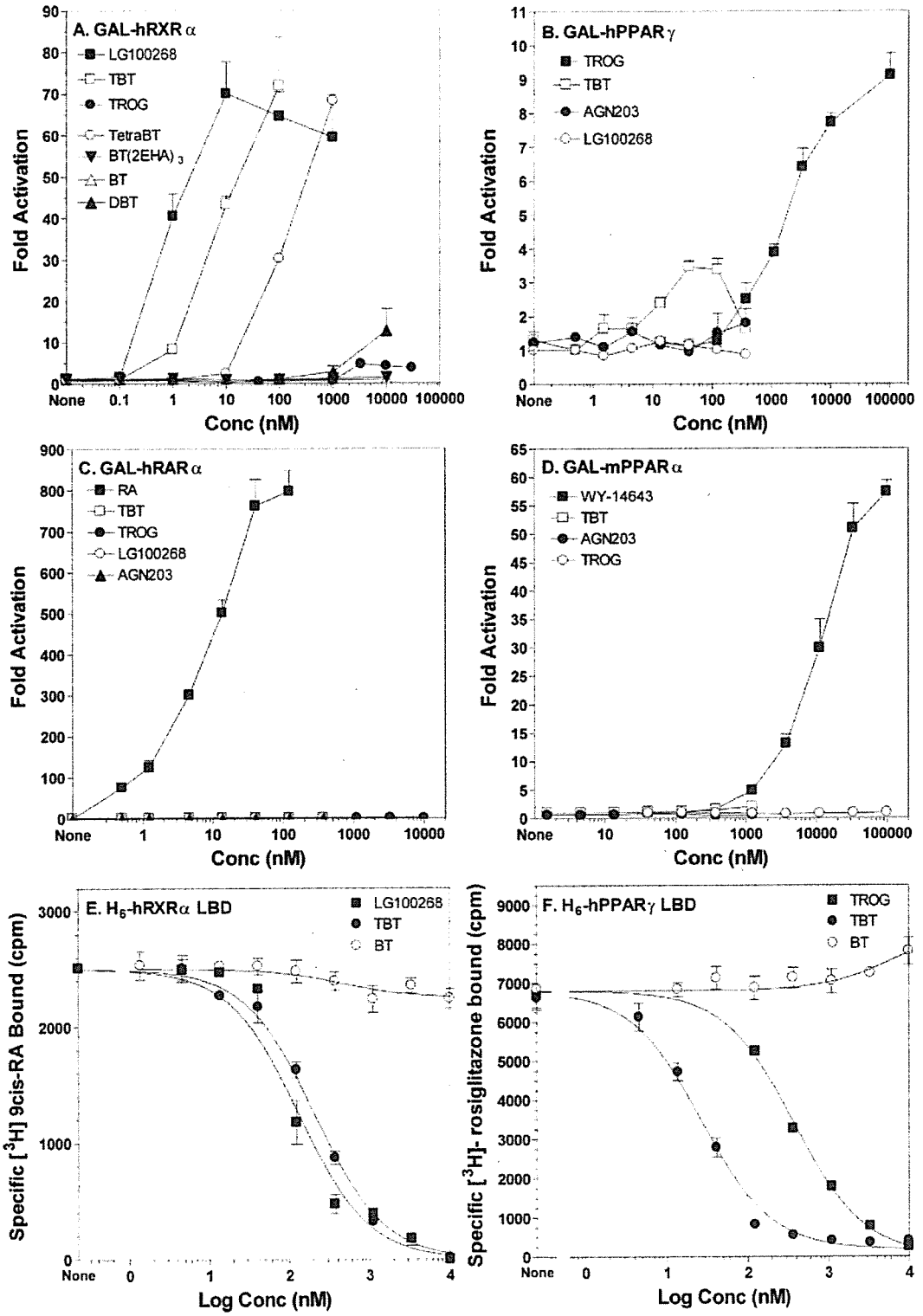

Fig. 1. Organotins Are Agonist Ligands of RXRα and PPARγ

Organotins are high-affinity ligand agonists of RXRα and PPARγ. A–D, Activation of GAL4-hRXRα, -hPPARγ, -hRARα, or -hPPARα in transiently transfected Cos7 cells by organotins and receptor-specific ligands. Data represent reporter luciferase activity normalized to β-galactosidase and plotted as the average fold activation ± SEM (n = 3) relative to solvent-only controls from representative experiments. E and F, Competition binding curves of histidine-tagged RXRα or PPARγ LBDs with TBT. Data shown are from a representative experiment analyzed in GraphPad Prism 4.0 and K_i values deduced (Table 3). Conc, Concentration; DBT, dibutyltin chloride; TROG, troglitazone.

Table 1. TBT Activates RXRs and RXR-Permissive Heterodimers

GAL4-NR LBD	Fold Activation at 60 nM TBT	Permissive RXR Heterodimer
RXRα (*Homo sapiens*)	60	Yes
RXRα (*X. laevis*)	25	Yes
RXRγ (*X. laevis*)	7.0	Yes
NURR1 (*H. sapiens*)	7.0	Yes
LXR (*H. sapiens*)	2.1	Yes
PPARα (*Mus musculus*)	0.7	Yes
PPARγ (*H. sapiens*)	5.3	Yes
PPARδ (*H. sapiens*)	1.7	Yes
RARα (*H. sapiens*)	0.7	No
TR&β (*H. sapiens*)	0.4	No
VDR (*H. sapiens*)	0.5	No
SXR (*H. sapiens*)	1.0	No

Data are fold activation at 60 nM TBT relative to solvent-only controls of transiently transfected Cos7 cells after 24 h ligand treatment. SXR, Steroid and xenobiotic receptor; TR, thyroid hormone receptor.

molar EC_{50} values. Monobutyltin gave no significant activation whereas dibutyltin was moderately active in the micromolar range (Fig. 1A and Table 2). Tetrabutyltin was 20-fold less potent than TBT, whereas the branched side-chain butyltin tris(2-ethylhexanoate) [BT(2-EHA)$_3$] was inactive (Table 2). Although activation by dialkyltins is weaker than that of TBT, it is potentially significant due to their widespread use in the manufacture of polyvinyl chloride plastics and greater solubility than TBT.

The effect of the hydrocarbon chain length was very pronounced, suggesting an important structure-activity relationship. A reduction in hydrophobicity from butyl to ethyl side chains raised the EC_{50} value by almost 1000-fold into the micromolar range. Trimethyltin was weakly active only above 100 μM (Table 2). Substitution of the halide component had no significant effect on the EC_{50} values for TBT, probably due to the lability of the halide atom through exchange in aqueous tissue culture media where chloride ions are prevalent.

Table 2. Organotin EC_{50} Values for Nuclear Receptor LBDs

Ligand	GAL4-NR LBD Transactivation (EC_{50} Values, nM)		
	hRXRα	hRARα	hPPARγ
LGD268	2–5	na	na
AGN195203	0.5–2	na	na
9-*cis* RA	15	na	na
all-*trans* RA	na	8	na
Butyltin chloride	na	na	na
Dibutyltin chloride	3000	na	na
TBT	3–8	na	20
Tetrabutyltin	150	ND	ND
Di(triphenyltin)oxide	2–10	na	20
Butyltin tris(2-ethylhexanoate)	na	ND	ND
Troglitazone	na	na	1000
Tributyltin fluoride	3	ND	ND
Tributyltin bromide	4	ND	ND
Tributyltin iodide	4	ND	ND
Triethyltin bromide	2800	ND	ND
Trimethyltin chloride	>10000	ND	ND

na, Not active; ND, not determined. EC_{50} values were determined from dose-response curves of GAL4-NR LBD construct activation in transiently transfected Cos7 cells after 24-h ligand exposure.

TBT Is a Potent Ligand of Both RXRα and PPARγ

Many, if not most, natural and synthetic nuclear receptor agonists act as ligands that specifically interact with their cognate receptor LBDs. We therefore performed equilibrium competition binding experiments with purified histidine-tagged human RXRα (H_6-RXRα) and PPARγ (H_6-PPARγ) LBDs to determine whether the potent and specific activation of these receptors by TBT was due to direct ligand-receptor interaction (Fig. 1, E and F).

The equilibrium binding curves indicate that TBT is a high-affinity, competitive ligand for 9-*cis* RA-bound RXRα. The inhibition equilibrium dissociation constant was calculated by the Chang-Prusoff method [inhibition constant (K_i) = dissociation constant (K_d)] as 12.5 nM (10–15 nM; 95% confidence interval) (Table 3). By comparison, the value obtained for the synthetic RXR agonist LG100268 was 7.5 nM, which compared favorably with its published value of approximately 3 ± 1 nM (27). Therefore, the identification of TBT as an RXR ligand expands the molecular definition of known rexinoids (agonists able to activate RXR) to include this structurally unique class of organotin compounds.

Somewhat surprisingly, we also observed potent specific competitive binding by TBT for rosiglitazone bound to human PPARγ LBD (Fig. 2B). The deduced K_i of 20 nM (17–40 nM; 95% confidence interval) was slightly higher than for RXRα but significantly better than the K_i for the PPARγ agonist troglitazone, which

Table 3. TBT Binding Constants (K_d) for hRXRα and hPPARγ LBDs

Ligand	Receptor Competitive Inhibition Binding Constants K_i (nM ± 95% CI)		
	H_6-RXRα	H_6-PPARγ	Published
TBT	12.5 (10–15)	20 (17–40)	
LG100268	7.5 (6–10)	ND	3 ± 1[a]
Troglitazone	ND	300 (270–335)	300 ± 30[b]

Competition binding curves were determined at constant ^{3}H-specific ligand concentrations [20 nM 9-*cis*-RA, K_d = 1.4 nM (87) or rosiglitazone, K_d = 41 nM (88)] with increasing cold competitor ligands over the range indicated in Fig. 1, E and F. Data were analyzed in GraphPad Prism by nonlinear regression of a competitive one-site binding equation (Chang-Prusoff method) to determine K_i values ± 95% confidence intervals (n = 3). CI, Confidence interval; ND, not determined.
[a] RXRα:LG100268 K_d = 3 ± 1 nM (27).
[b] PPARγ:troglitazone K_d = 300 ± 30 nM (28).