data for an epigenetic reprogramming mechanism and for the environmental health risk assessment.

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Rat Thyroid Hyperplasia Induced by Gestational and Lactational Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin

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Effects of gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on thyroid function of offspring were investigated in the rat. Pregnant Holtzman rats, TCDD-sensitive strain, were given a single oral dose of 200 ng or 800 ng TCDD/kg on gestational day 15. Parameters related to the thyroid functions were examined on postnatal days (PNDs) 21 and 49. Serum T_4 levels in offspring decreased significantly on PND21 in the two TCDD-exposed groups but increased on PND 49 only in the high-dose group. A dose of 800 ng TCDD/kg exerted a more than 2-fold increase in serum TSH level in male offspring on PNDs 21 and 49. A significant induction of uridine diphosphate-glucuronosyltransferase-1 gene by TCDD was observed on PND 21 but returned to basal levels on PND 49. Gene expression of cytochrome P4501A1 was markedly induced in the liver treated with TCDD. Even a single oral perinatal exposure to 800 ng TCDD/kg resulted in hyperplasia of the thyroid gland of offspring on PND 49. Proliferating cell nuclear antigen immunocytochemistry also supported this finding. Thus, gestational and lactational exposure to TCDD was found to disrupt thyroid hormone homeostasis, which results in a sustained excessive secretion of TSH, followed by the hyperplasia of thyroid follicular cells. (*Endocrinology* 144: 2075–2083, 2003)

DIOXIN AND DIOXIN-LIKE polychlorinated biphenyls (PCBs) are ubiquitously present in the environment, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent isomer among the large family of dioxin and related compounds. In addition to reproductive, immunological, neurobehavioral toxicities, teratogenicity, and carcinogenicity, the alteration of thyroid hormone metabolism and function by TCDD has been published. Thyroid hormone is required for brain development, neuronal maintenance, and metabolic function during fetal and early neonatal periods (1). In adult rodents, exposure to TCDD resulted in morphological and functional alterations in the thyroid (2–5). Morphologically, TCDD exposure induced an increase in the volume of thyroid follicular cells, followed by a hyperplasia (6) and, eventually, follicular thyroid tumor in male Osborne Mendel rats (7). Exposure to TCDD also causes hypothyroxinemia by elevated excretion of T_4 into bile after the induction of uridine diphosphate (UDP)-glucuronosyltransferase-1 (UGT1). This is accompanied by an increase in TSH-positive cells in the anterior pituitary, followed by a foamy change in the colloid of some follicles, an indication of accelerating the biosynthesis of T_4 in the thyroid (8). Thus, a TCDD-caused induction of UGT1, an enzyme catalyzing T_4-glucuronide formation, results in the enhanced biliary excretion of T_4, which results in a reduced serum T_4 level (9, 10). It is established that reduction of circulating T_4 levels can accelerate TSH release by the negative feedback mechanism in the pituitary-thyroid axis, which, in turn, leads to compensatory production of T_4 in the thyroid gland.

In human populations, dioxin, dioxin-like PCBs, and other chemicals (particularly, non-dioxin-like PCBs), all of which are ubiquitous in the environment, may affect thyroid metabolism as well as neurobehavioral development. A Dutch cohort study suggested that maternal exposure to dioxin and/or PCBs, as represented as concentrations in breast milk, was associated with an increased level of TSH and a decreased level of T_4 in the serum of 105 children but not always for T_3 (11, 12). Furthermore, the follow-up of the Dutch cohort study (42-month-old infants) found an association between decreased cognitive ability and PCB concentrations in the maternal blood at birth and that attention and activity in these preschool children might be impaired by prenatal, as well as postnatal, PCB exposure (Ref. 13; no dioxin concentrations in the infants were available because approximately 100 ml of blood specimens are needed for dioxin determinations). It is important to perform a mechanistic study to examine the possible effects of dioxin and PCBs on thyroid morphology and function.

In a study on Long-Evans rats, approximately 0.05% of the administered dose to dams was transferred to a fetus (14). Administration of a mixture of PCBs, such as Aroclor 1254, to pregnant Long-Evans rats affected thyroid hormone levels of offspring, and postnatal T_4 replacement was shown to

Abbreviations: AhR, Arly hydrocarbon receptor; CYP1A1, cytochrome P4501A1; GC/MS, gas chromatograph/mass spectrometer; GD, gestational day; IHC, immunohistochemical; NIES, National Institute for Environmental Studies; PCB, polychlorinated biphenyl; PCNA, proliferating cell nuclear antigen; PND, postnatal day; RT, reverse transcription; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UDP, uridine diphoshate; UGT, UDP-glucuronosyltransferase-1.

attenuate hearing loss and motor deficit (15). Investigations on toxicological effects of gestational and lactational exposure to TCDD on thyroid of offspring are limited, however. By administering TCDD at a daily dose of 0.1 μg/kg to Sprague Dawley rats during gestational days (GDs) 10–16, Seo *et al.* (16) demonstrated a slight decrease in plasma T_4 concentration but not in T_3 and TSH concentrations on postnatal day (PND) 21; they concluded that the degree of T_4 suppression observed was too small to be clinically significant. Regarding carcinogenicity of TCDD, oral administration of TCDD, twice a week for 2 yr, resulted in follicular cell adenomas in female B6CF1 mice and male and female Osborne-Mendel rats in a dose-related fashion (17).

Because not many other studies showed carcinogenicity, particularly in the thyroid, there seems a remarkable difference in susceptibility to TCDD exposure among strains and/or species. Regarding lethality, the guinea pig is the most sensitive, the rat and mouse have intermediate sensitivity, whereas the hamster is relatively tolerant (18). The effective dose to induce changes in thyroid hormone levels in hamsters is higher than that in rats (19). Weber *et al.* (20) examined differences in sensitivity to TCDD toxicity using the TCDD-susceptible (C57Bl/6J) and the TCDD-less sensitive (DBA/2) mouse strains, and showed that doses of TCDD required to affect thyroid hormone levels were about 1000 times lower in C57Bl/6J mice than in DBA/2 mice. In the rat, a marked difference in responsiveness to TCDD has been shown by using CTP1A1 as a marker gene, with the Sprague Dawley rats being the least sensitive strain, in contrast to the higher sensitivity of the Holtzman rat strain (21).

In the present study, we evaluated the gestational and lactational effects of TCDD on offspring, by administering a single oral dose of TCDD to pregnant Holtzman rats on GD 15, and examined, on PNDs 21 and 49, a variety of parameters related to thyroid functions as well as morphology. Unexpectedly, we have found that this dosing regimen produced hyperplasia in the thyroid of the TCDD-exposed male and female offspring.

Materials and Methods

Animals and exposure

Male and female Holtzman rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and bred at the National Institute for Environmental Studies (NIES, Tsukuba, Japan). They were maintained in controlled room at 23 ± 1 C and humidity at 50 ± 10% on a 12-h light, 12-h dark cycle. The animals received food and distilled water *ad libitum* and were handled with humane care under the guidelines of the NIES. Ten-week-old female rats in proestrus were mated 1:1 with males overnight, and females that had a vaginal plug the following morning were designated as d 0 of gestation. Pregnant rats (six per group) were dosed by gavage with corn oil (vehicle) and 200 or 800 ng TCDD/kg on GD 15. On PND 2, litters were randomly culled to eight offspring, with five males and three females, when possible. On PNDs 21 or 49, tissues (including liver, pituitary glands, and thyroid glands with the trachea) were excised from offspring anesthetized lightly with diethyl ether. Tissues were fixed in Zanboni's solution (22) for 24 h at 4 C and processed for immunohistological examinations. For biochemical examination, tissues were snap-frozen in liquid nitrogen and stored at −80 C until analysis.

Immunohistochemistry

T_4 and proliferating cell nuclear antigen (PCNA) in the thyroid and TSH in the pituitary gland were stained in tissue sections by an indirect immunohistochemical (IHC) technique (23, 24). Briefly, the deparaffinized and rehydrated sections were pretreated in 0.01 M sodium citrate buffer, pH 6.0, with microwave heating and washed in PBS. To quench endogenous peroxidase activity, sections were covered with 0.3% H_2O_2 dissolved in 100% methanol. Rabbit antibody against T_4 (RP 039; Diagnostic BioSystems, Pleasanton, CA) was diluted 1:200, and monoclonal mouse anti-PCNA (Clone PC 10, Code No. M 0879; DAKO Corp., Glostrup, Denmark) was diluted 1:300 or rabbit antibody against TSH (AB976; Chemicon International, Inc., Temecula, CA) was diluted 1:1000 in PBS. The primary antibodies were incubated over the sections in a humidified chamber for either 60 min at 25 C for T_4 staining or 30 min at 37 C for TSH and PCNA immunostaining. Sections were subsequently washed with PBS, followed by incubation for 1 h with biotinylated goat antirabbit IgG (BA-1000; Vector Laboratories, Inc., Burlingame, CA) or antimouse IgG (BA-9200) that were diluted 1:200 in PBS. They were incubated with an avidin-biotinylated peroxidase complex (PK-4000; Vector Laboratories, Inc.). Immunoreactions were performed by using hydrogen-peroxide-activated 3,3′-diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, MO). Sections were counterstained for 10 sec in Mayer's hematoxylin. Negative controls, in which the primary antibody was replaced with normal rabbit IgG, did not show nonspecific staining. PCNA-positive cells were quantified by counting the number of cells per low-power field for three fields, by the use of an Image Processing and Analysis System (Version 2.2A; Leica Corp., Qwin, Cambridge, UK). The degree of thyroid alteration was also quantified by applying the system to hematoxylin and eosin-stained thyroid tissue sections. The follicular lumenal, parenchymal, and total areas were quantified, and the values reported for each animal represent means of three random fields per each tissue slice.

Thyroid hormone analyses

Serum total T_4 and total T_3 levels were determined with Amerlex RIA kits (Amersham International, Buckinghamshire, UK), according to the manufacturer's instructions (6). Serum TSH levels were determined with a rat TSH enzyme immunoassay kit (Amersham International).

RNA extraction and RT-PCR

Total hepatic RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan). Enzymes and cofactors for reverse transcription (RT) and PCR were purchased from Takara (Otsu, Japan). All the primers used in the present study were purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ). Sequences of PCR primers for amplification of cytochrome P4501A1, UGT1 (the common region of UGT1 isoforms), and β-actin were the same as reported earlier (25). RT of RNA was performed in a final vol of 20 μl solution containing 5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphate, 0.25 U/μl avian myeloblastoma virus reverse transcriptase, 0.125 μM oligo dT-adaptor primer, 1 U/μl ribonuclease inhibitor, and 1 μg total RNA, using the RNA LA PCR kit (Takara). The RT samples were incubated at 42 C for 15 min and then at 99 C for 5 min for inactivation of reverse transcriptase. PCR was subsequently performed as follows: reaction mixture was incubated at 94 C for 4 min and then amplified at 94 C for 30 sec, 56 C for 30 sec, and 72 C for 1 min. Reactions were repeated for 21 cycles for UGT1 and 22 cycles for β actin and cytochrome P4501A1 (CYP1A1). The PCR mixture (10 μl) contained 2.5 mM $MgCl_2$, 0.25 U Takara LATaq, 0.2 μM each forward and reverse primer, and 2 μl RT products. PCR products were detected as a single band on 1.5% agarose gel in 1× Tris-borate EDTA containing 2 μg/ml ethidium bromide. Band intensity was quantified by the EDAS120 system (version 2.02; Eastman Kodak Co., Rochester, NY).

TCDD analysis

TCDD in tissue specimens (serum, liver, and adipose tissues) was determined by essentially the same method as described earlier (23). In brief, samples collected and pooled from five rats were weighed and spiked with ^{13}C-2,3,7,8-TCDD (Wellington, Ontario, Canada) as the internal standard. The tissue was digested and washed, and TCDD was extracted with n-hexane. The n-hexane layer was cleaned by concentrated sulfuric acid, rinsed with water, and dried. The solution was concentrated and cleaned further by silica gel (Kieselgel 60; Merck KGaA, Darmstadt, Germany) and activated carbon-silica gel (active

carbon impregnated-silica gel; Wako Pure Chemical Industries Ltd., Osaka, Japan) column chromatography.

The gas chromatograph/mass spectrometer (GC/MS) analysis was performed in the selected ion mode on a JMS700 high-performance double-focusing mass spectrometer (JEOL Inc., Tokyo, Japan) coupled to an HP 6890 gas chromatograph (Hewlett-Packard Co., Wilmington, DE). Sample solution was introduced into an HP 6890 equipped with a CP-SIL 8CB/MS column (Chrompack, EA Middelburg, The Netherlands; 30 m × 0.25 mm inside diameter, film thickness 0.25 μm). Identification was based on the correct isotope ratio of M^+ to $(M + 2)^+$ (±15%), recoveries (50–120%), and retention time (±4.0 sec) of the GC separation. The area of mass profile peaks of the quantification ions was used for the quantitative analysis of TCDD. Quantified values were calculated by the internal standard methods.

Statistical analysis

StatView for Windows (version 5.0; SAS Institute, Inc., Cary, NC) was used for statistical analysis. Data are expressed as mean ± SEM. Differences in means among the three groups were analyzed by one-way ANOVA followed by Scheffé's test as *post hoc* comparison. Comparisons of mean values between males and females were performed by Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Results

Reproductive and developmental outcomes

No signs of toxicity were observed in dams treated with TCDD at a dose of 200 ng/kg (low-dose group), whereas a significant decrease in litter size was observed in the 800-ng TCDD/kg (high-dose group; Table 1). TCDD treatment did not cause statistically significant changes in sex ratios and birth weights of offspring. Body weight, brain weight, and thyroid (right lobe) weight of the offspring at the time of weaning were not affected by the treatment (data not shown).

Thyroid hormone concentrations

Administration of TCDD to dams on GD 15 decreased (in a dose-dependent manner) total T_4 concentrations in the serum from both male and female offspring on PND 21 (Fig. 1A). In particular, the T_4 concentrations were significantly lower in male offspring in both the low- and high-dose groups than those in vehicle-treated offspring. Conversely, on PND 49, the serum T_4 concentration of male offspring in the high-dose group was significantly increased (Fig. 1A). A similar trend was observed in the female rats in the high-dose group, although the increase was not statistically significant. Serum T_3 concentration was significantly increased on PND 21 in female offspring in the high-dose group, compared with the vehicle-treated control, but no statistical difference was observed on PND 49 (Fig. 1B).

This elevated level of biosynthesis of T_4 in response to TCDD was confirmed immunohistochemically in the offspring thyroid gland. On PND 49, the marked induction of T_4 was clearly shown in the male thyroid glands of the high-dose group. The staining intensity for T_4 was stronger in the colloid lumina of follicles in male and female TCDD-treated offspring, compared with the vehicle-treated controls (Fig. 2, A–D). The cytoplasm of most of the follicular cells was negative at this time. However, no significant change in the localization pattern for T_4 was found in male and female thyroid glands on PND 21 (data not shown). In these offspring, T_4 was positively stained in the cytoplasm of follicle epithelial cells and weakly and homogeneously in the colloid lumina of follicles. Because the localization of T_4 is very similar to that of thyroglobulin (22), the detection of T_4 immunoreactivity found in the present study is thought to reflect the localization of both non-Tg-bound and Tg-bound T_4.

The serum TSH concentrations in male and female offspring were significantly increased in the high-dose group, in comparison with the vehicle-treated control, and remained elevated until PND 49 (Fig. 3). The IHC staining for TSH confirmed the induced biosynthesis of TSH by TCDD in the anterior pituitary gland of male and female offspring. The staining intensity and the number of positive cells for TSH

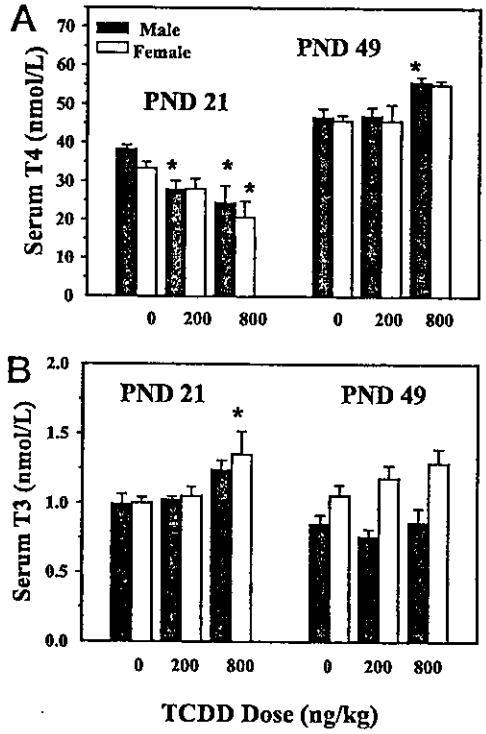

FIG. 1. Effects of gestational and lactational exposure to TCDD on serum levels of T_4 (A) and T_3 (B) on PNDs 21 and 49. Values are mean ± SEM (n = 4–6). *, Significantly different from vehicle-treated controls ($P < 0.05$).

TABLE 1. Reproductive outcomes of dams exposed to TCDD on GD15

Dose of TCDD (ng/kg bw)	Litter size			Offspring birth weight (g)	
	Total	Male	Female	Male	Female
0	11.0 ± 0.9	4.6 ± 1.1	6.4 ± 1.2	9.3 ± 0.8	9.1 ± 0.6
200	11.8 ± 1.1	7.3 ± 1.9	4.5 ± 1.8	8.7 ± 0.5	8.1 ± 0.6
800	4.3 ± 0.3[a]	2.0 ± 0.0	2.3 ± 0.3[a]	8.7 ± 0.7	8.2 ± 0.7

Data are mean ± SEM for 5 rats. bw, Body weight.
[a]Statistically significant difference ($P < 0.05$) from vehicle-treated group, by ANOVA with a *post hoc* Scheffé's test.

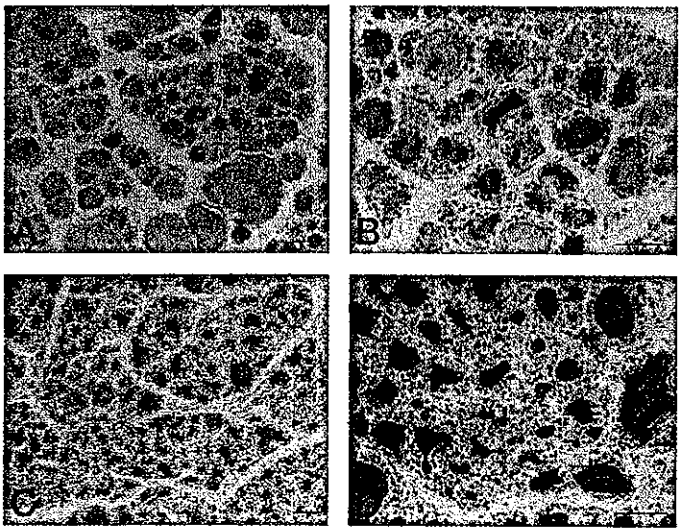

FIG. 2. IHC detection of T_4 in thyroid glands of male offspring, on PND 49, after gestational and lactational exposure to TCDD. A, Thyroid gland from a vehicle-treated offspring. The follicles with or without immunostaining for T_4 were dispersed in the thyroid gland. B, High magnification of A. T_4 localized in the follicular colloids but not in the follicular epithelial cells. C, Thyroid gland from offspring after gestational and lactational exposure to 800 ng TCDD/kg. An increased intensity of immunostaining in the thyroid follicles was noted. Intensely stained thyroid follicles were dispersed in thyroid gland, which was characterized by smaller size and an increase in total number of follicles, compared with the vehicle-treated control. D, High magnification of C. Strong T_4 immunostaining was observed mainly in the follicular colloid. *Bar*, 50 μm.

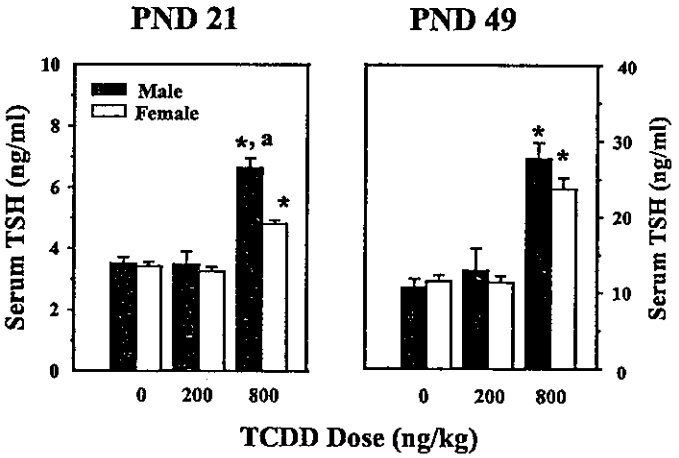

FIG. 3. Effects of gestational and lactational exposure to TCDD on serum levels of TSH on PNDs 21 and 49. Values are mean ± SEM (n = 4–6). *, Significantly different from vehicle-treated controls ($P < 0.05$); a, significantly different from female rat (Student's t test, $P < 0.05$).

were increased in the anterior pituitary of TCDD-exposed offspring on PND 21 (Fig. 4B) and PND 49 (Fig. 4D).

UGT1 and CYP1A1 mRNA in the liver

UGT1 and CYP1A1 mRNA levels in the liver of offspring were quantified by RT-PCR (Fig. 5). On PND 21, a significant increase in the UGT1 mRNA level was observed in male and female offspring in the low- and high-dose groups ($P < 0.05$), but differences were not longer apparent on PND 49 (Fig. 5C). However, a marked induction of CYP1A1, attributable to TCDD, was found in the liver of male and female offspring on both PNDs 21 and 49 (Fig. 5B).

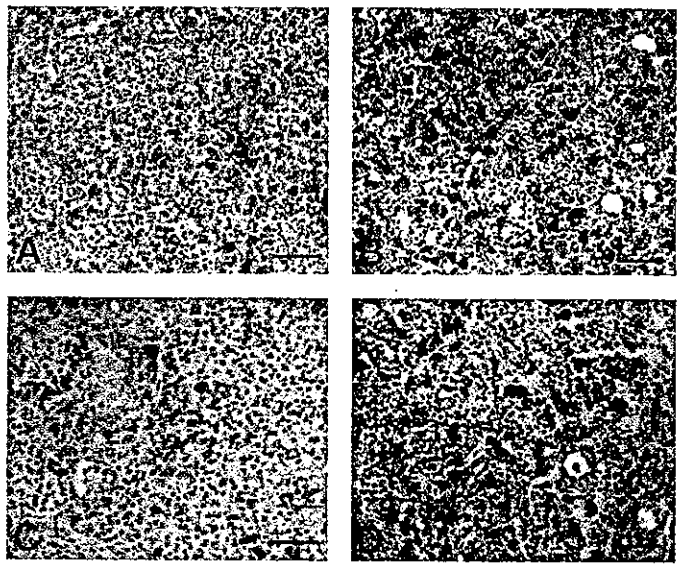

FIG. 4. IHC detection of TSH in the pituitary gland of male offspring after gestational and lactational exposure to 800 ng TCDD/kg. Note an increased intensity of immunostaining and the number of positive cells for TSH in the anterior pituitary of the TCDD-treated rat, compared with the vehicle-treated control. A, Pituitary gland from a vehicle-treated offspring on PND 21. B, Pituitary gland from a TCDD-treated offspring on PND 21. C, Pituitary gland from a vehicle-treated offspring on PND 49. D, Pituitary gland from a TCDD-treated offspring on PND 49. *Bar*, 50 μm.

TCDD concentrations in the tissues

TCDD concentrations in male offspring tissues are summarized in Table 2. We analyzed TCDD concentrations in a single specimen for each dose group and for each time point by combining tissue specimens from six rats, because dioxin determination by high-resolution GC-MS was extremely laborious and costly. Consistent with an earlier study (16), large amounts of TCDD were found in the offspring liver and adipose tissue on PND 21. The tissue TCDD concentration had decreased markedly by PND 49, for example, to almost 2% of that of the liver on PND 21. The TCDD concentration was reversed between the liver and adipose tissue, particularly in the high-dose group, on PND 49.

Thyroid histology

From hematoxylin and eosin staining, maternal exposure to 800 ng TCDD/kg (high-dose group) resulted in the diffuse hyperplasia of follicular cells in the male rat thyroid on PND49 (Fig. 6C), in contrast to no apparent changes in vehicle-treated rat thyroid (Fig. 6, A and B). A high-power view of thyroid tissues (Fig. 6D) demonstrated that the TCDD-exposed thyroid exhibited hyperplasia, characterized by small follicles and tall columnar epithelium having a slightly larger nucleus. In addition to hyperplasia in the follicular epithelium, TCDD exposure produced an increase in the number of abnormally shaped follicles, as well as fibroplastic lesions in the interstitial connective tissue.

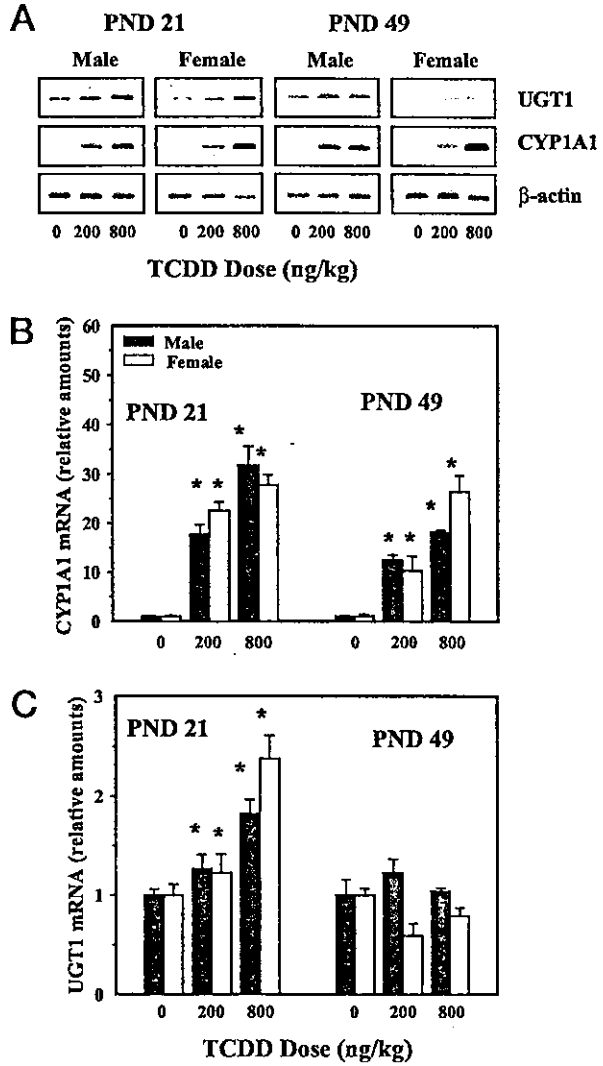

FIG. 5. Dose-response of CYP1A1 (A and B) and UGT1 (A and C) mRNA expression in livers of male and female offspring, on PNDs 21 and 49, after perinatal exposure to TCDD, by RT-PCR analysis. β-actin (A, *bottom panel*) was used to check for equal loading of mRNA. Values are mean ± SEM (n = 4–6). *, Significantly different from vehicle-treated controls ($P < 0.05$).

TABLE 2. Distribution of TCDD in liver, adipose, and serum of offspring, on PNDs 21 and 49, after gestational and lactational exposure to TCDD

	TCDD (pg/g tissue)					
TCDD dose (ng/kg bw)	PND 21			PND 49		
	Liver	Adipose	Serum	Liver	Adipose	Serum
0	0.3	[a]	<0.1	0.1	0.4	<0.1
200	418	519	11.4	20.8	90.3	0.4
800	2250	1329	59.6	55.3	209	4.0

Liver, adipose, and serum were collected from six rats and combined. The amounts of TCDD were determined by GC-MS. bw, Body weight.

[a] Not performed.

The thyroid of 49-d-old female offspring responded to TCDD in a slightly different manner than that in male offspring (Fig. 6, E and F). The hyperplasia in the female offspring was not as prominent (Fig. 6, E and F), although many small follicles are similarly observed. Hyperplastic areas, quantitatively analyzed in terms of the ratio of parenchymal area to follicular area, showed that the ratio was significantly increased in both male and female offspring in the high-dose group (Fig. 6G). In addition, the area ratio (parenchymal/luminal) of thyroid was significantly higher in male offspring than female offspring.

PCNA immunocytochemistry was performed to assess the proliferative action of TCDD in the thyroid gland of 49-d-old male offspring. Follicular cells that had PCNA-labeled nuclei were found in vehicle-treated control rat thyroid (Fig. 7A), whereas a drastic increase in number of PCNA-positive cells was found in the offspring thyroid from the high-dose group (Fig. 7B), indicating an ability of TCDD to induce cell proliferation. When the ratio of PCNA-positive cells to total number of follicular cells was calculated and compared between TCDD-treated offspring and vehicle-treated control offspring on PND 49, we found a significant increase in the ratio in the thyroid from both male and female offspring in the high-dose group (Fig. 7C). There was a tendency for a much higher frequency of PCNA-labeled nuclei to be observed in males than in females ($P < 0.05$).

Discussion

The most remarkable, but unexpected, finding in the present study is that even a single oral administration of TCDD to pregnant rats was sufficient to produce a hyperplastic alteration in the thyroid of both male and female offspring on PND49. The hyperplasia, which was confirmed by morphometric analysis (Fig. 6G), was characterized by small follicles with cubical and columnar epithelium with an elevated number of PCNAs (Fig. 7), which was also observed in adult animals exposed to TCDD and many goitrogenic xenobiotics. For example, Sewall *et al.* (6) found similar morphological changes in the adult rat thyroid after a 30-wk continuous treatment with TCDD; they suggested that prolonged stimulation of the thyroid by TSH is responsible for these changes. Although no thyroid neoplasia or nodules were observed after TCDD treatment in the present study, the diffuse thyroid hyperplasia induced by continued stimulation by TSH might progress to nodules and eventually neoplasia (see review in Ref. 26). These data, as well as the present observations, support the notion that long-term perturbations of the pituitary-thyroid axis by xenobiotics or physiological alterations are likely to predispose rats to a higher incidence of proliferative lesions (27).

We found that perinatal exposure to TCDD caused a marked decrease in the circulating T_4 level at weaning (PND 21), in a dose-dependent manner (Fig. 1A), and serum TSH level was increased significantly on PNDs 21 and 49 in the high-dose group (Fig. 3). We also observed a significant correlation between induction of UGT1 gene expression and suppression in serum T_4 levels. Although the elevated level of UGT1 mRNA did not last until PND 49 (Fig. 5), it is possible that a drastic decline of hepatic TCDD concentration, approximately 2% of the value in the rat liver on PND 21 (Table 2), is responsible for this phenomenon. This perturbation of thyroid hormone balance in the TCDD-exposed

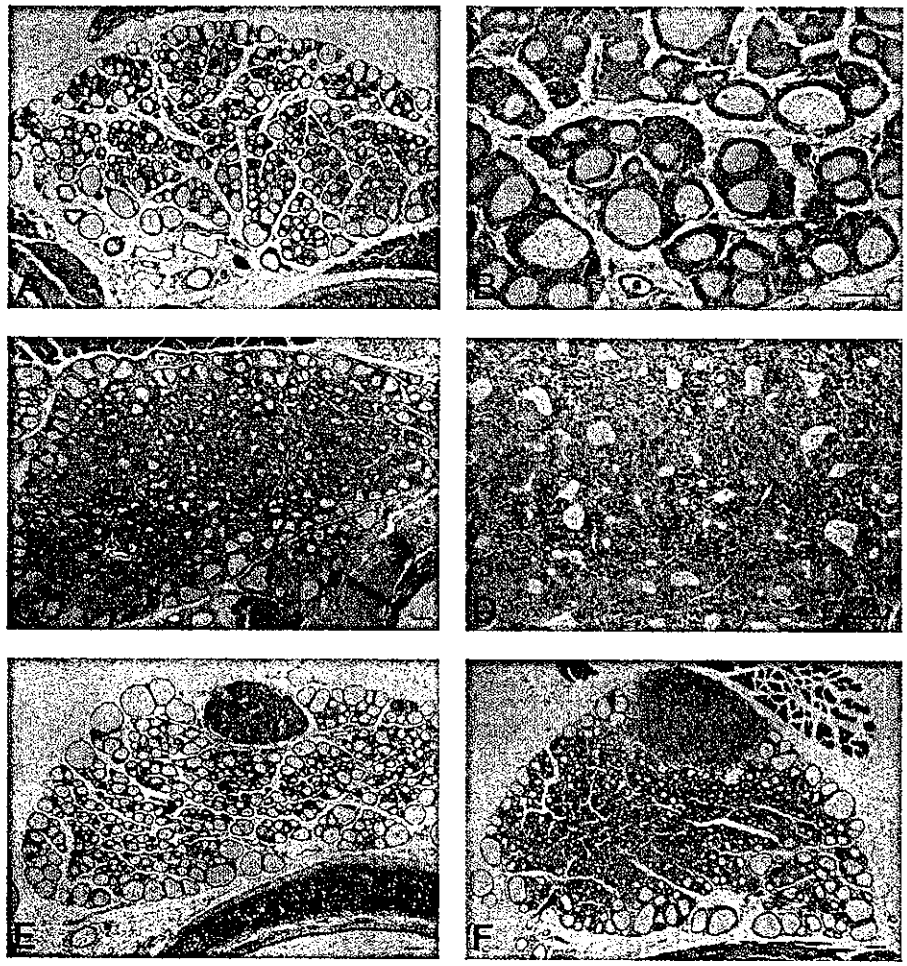

FIG. 6. Histological comparison of offspring thyroid glands, on PND 49, after gestational and lactational exposure to TCDD. A, Thyroid gland from a male vehicle-treated offspring. B, High magnification of A. C, Thyroid gland from a male offspring after gestational and lactational exposure to 800 ng TCDD/kg. Note the increase in the number of small follicles, compared with A. D, High magnification of C, showing small follicles and abnormally shaped follicles as well as fibroplastic lesion in interstitial connective tissue. E, Thyroid gland from a female vehicle-treated offspring. F, Thyroid gland from a female TCDD-treated offspring. Note the many small follicles in thyroid glands, but hyperplastic capability was not as prominent in females as in males. Sections were stained with hematoxylin and eosin. *Bar*, 100 μm for A, C, E, and F; 50 μm for B and D. G, Quantification of hyperplastic changes in thyroid gland by measuring the ratio of parenchymal area to thyroid lumenal area. Values are mean ± SEM (n = 4–6). *, Significantly different from vehicle-treated controls ($P < 0.05$); a, significantly different from female rat (Student's t test, $P < 0.05$).

offspring is thought to be caused by the enhanced excretion of T_4-glucuronide via induction of UGT1 in response to TCDD, as has been reported for TCDD-exposed adult animals (5). Because TCDD is reported to induce UGT1A6 (28), it is reasonable to speculate that UGT1A6 is primarily responsible for T_4 glucuronidation in offspring after maternal TCDD exposure in the present study. Vansell *et al.* (29) also suggested that PCB (Aroclor 1254) enhanced T_4 UGT activity by increased expression of UGT1A6. Recently, using an antibody against UGT1A6- or UGT1A1-specific peptide, we observed an induction of UGT1A6, but not UGT1A1, in the centrilobular region of the liver of TCDD-exposed rats (30). Among nine isoforms of UGT1A family, TCDD-induced UGT1A6 is thought to glucuronidate T_4. In our recent study, we have found an elevation of UGT1A7 mRNA in the liver of rats perinatally exposed to TCDD (30), which may suggest that UGT1A7 might be responsible for glucuronidation of T_4.

Circulating T_3 concentrations were observed to be elevated in female offspring in the high-dose group on PND 21 but not on PND 49. Because a single dose of TCDD was reported to decrease in type I deiodinase activity in a dose-dependent manner in adult rats (31), it is not likely that type I deiodinase

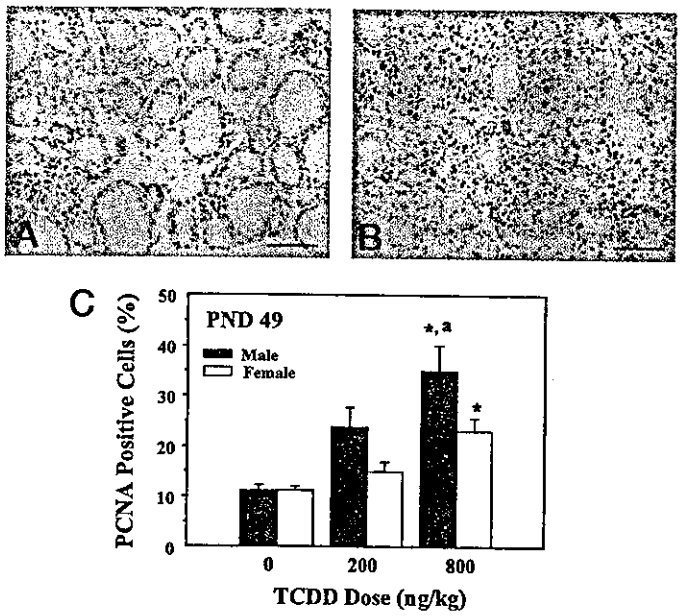

FIG. 7. PCNA staining in thyroid glands from male offspring, on PND 49, after gestational and lactational exposure to 800 ng TCDD/kg. A, Thyroid gland from a vehicle-treated offspring. B, Thyroid gland from a TCDD-treated offspring. Note the dramatic increase in the number of PCNA-positive cells in the thyroid gland. C, The ratio of PCNA-positive cells to total number of thyroid follicular cells. *, Significantly different from vehicle-treated controls ($P < 0.05$); a, significantly different from female rat (Student's t test, $P < 0.05$).

is associated with the above-mentioned increase in the serum T_3 in the present study. Because sulfation is responsible for the elimination of T_3 from liver to the bile (26), it is worth studying whether and how TCDD might affect this metabolic pathway.

Next, we addressed questions of whether or not this perturbation in the hypothalamus-pituitary-thyroid axis is mediated by an aryl hydrocarbon receptor (AhR)-dependent mechanism and which period of exposure (gestational or lactational) is more responsible for this perturbation. In a previous study (32), we administered a single oral dose of TCDD to pregnant AhR knock-out mice on GD 12. No induction of UGT1 gene expression by TCDD administration in offspring (PND 21) was observed, indicating that this induction was dependent on AhR. To clarify the critical exposure period in terms of perturbation of the thyroid homeostasis by TCDD, a cross-fostering study might answer the question. Earlier studies suggested that there may be a different critical window period for each endpoint (33, 34). Recently, we have found that lactational, rather than gestational, exposure to TCDD plays a crucial role in disrupting the thyroid hormone balance (30).

It has been established that circulating thyroid hormone levels, T_3 and T_4, are regulated by the negative feedback mechanism in the hypothalamus, which produces TRH, and in the pituitary, which secretes TSH, leading to the control of circulating T_3 and T_4 concentrations. Some explanations may be drawn from epidemiological and laboratory studies. Serum T_3 and T_4 levels were reported to be significantly higher in Yusho patients who had accidentally ingested rice oil that contained PCBs, polychlorinated dibenzofurans, and polychlorinated quaterphenyls; and several Yusho patients had elevated serum TSH levels (35). In a Dutch epidemiologic study (36), 11-wk-old infants who were breast-fed with a relatively higher background level of dioxin and PCBs in their mothers' milk were found to have higher levels of serum total T_4, T_4/T_4-binding globulin ratio, and TSH, which led to the conclusion that these compounds modulate the hypothalamic-pituitary-thyroid regulatory system in the newborns. The coincidentally induced levels of T_4 and TSH in the rat, on TCDD exposure (Figs. 1 and 3), were supported by the enhanced biosynthesis of TSH and T_4 by TCDD in the target organs, pituitary and thyroid, respectively (Figs. 2 and 4), and may be relevant to the above-mentioned observations in humans. An alternative explanation for coincidental increases in T_4 and TSH levels would be that TCDD disrupts the negative feedback mechanism in the hypothalamic-pituitary-thyroid axis, which results in secretion of pituitary TSH independently of serum levels of thyroid hormones.

We found a sex difference in the degree of development of hyperplasia and an alteration of thyroid hormone status (Fig. 3). Male rats developed a higher degree of hyperplasia and a higher concentration of serum TSH than female rats in the high-dose group, suggesting that female offspring were not as sensitive to TCDD exposure as male offspring (Figs. 6 and 7). A sex difference in thyroid tumorigenesis was reported by Hiasa *et al.* (37), in which promotion of thyroid tumors by phenobarbital was found to be more conspicuous in male rats than female rats. McClain *et al.* (38) reported that nitrosamine administration significantly induced thyroid tumors in male rats, compared with no tumors in female rats; they attributed this sex difference in tumor induction to the lower basal level of TSH in female rats, compared with male rats. A plausible explanation would be a difference in basal TSH concentrations between male and female rats in their study, but this explanation cannot be applied to the present study because no sex differences in the basal level of TSH were observed. It should be noted that, in a carcinogenicity study by the U.S. National Toxicology Program (17), Osborne-Mendel rats given gastric instillations of TCDD twice a week for 104 wk showed follicular-cell adenomas of the thyroid, both in males and females. Thus, no clear-cut explanation is possible for the sex difference in thyroid tumorigenesis observed in the present study.

The serum T_4 concentrations after TCDD exposure in Holtzman rat offspring in the present study differed from the data from a previous report in which TCDD was orally administered to Sprague Dawley rats on GDs 10–16 (16), in which there was a slight decrease in plasma T_4 concentrations in female offspring but not in male offspring. In addition, plasma T_3 and TSH concentrations were not affected in any of the groups (16). A difference in dosing protocol, 0.025 or 0.10 μg/kg/d for 7 d, as well as a strain difference in sensitivity to TCDD, may (at least partly) explain this apparent inconsistency. The Holtzman strain is classified as a high responder, whereas the Sprague Dawley strain is considered to be a low responder, in terms of CYP1A1 induction (21, 39, 40).

The question arises as to whether and how the present experimental results are relevant to human findings. As mentioned earlier, our cross-fostering study showed that lacta-

tional (rather than gestational) exposure to TCDD played a crucial role in disrupting the thyroid hormone homeostasis in Holtzman rats (32). In the general population, breast-fed infants exposed to higher dioxin-TEQ levels had lower plasma T_4 and higher TSH levels, at 2 wk after birth, in a Dutch cohort study (11); and dioxin-TEQ in breast milk was found negatively correlated with serum T_4 level of infants in Japan (41). It has been reported that in industrialized countries, newborns were exposed to approximately 25-fold higher levels of dioxin-TEQ (for example, 112 *vs.* 4 pg TEQ/kg as tolerable daily intake) (42). Although the rat thyroid harbors transthyretin other than T_4-binding globulin as a T_4 carrier protein and is thought to be more susceptible than human thyroid in the development of hyperplasia caused by the shorter plasma half-life of T_4 (43), it is still plausible to speculate that both human and rodent newborns are relatively at risk by being exposed to substantial amounts of dioxins via breast milk.

In conclusion, perinatal exposure to a low dose of TCDD was demonstrated to disrupt thyroid morphology and functions in offspring, leading to a hyperplasia of the thyroid gland, with a modulation of negative feedback mechanism through the hypothalamic-pituitary-thyroid axis. Whether this perturbation affects the development of brain functions warrants additional study.

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—Original—

Alterations of Gene Expression in Adult Male Rat Testis and Pituitary Shortly After Subacute Administration of the Antiandrogen Flutamide

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Abstract. In the course of profiling alterations of gene expression in the male reproductive system induced by anti-androgenic agents, 28 genes expressed in the testis or pituitary of adult rats were examined shortly after subacute administration of the well-known anti-androgen, flutamide (FM). FM (25 mg/kg/day) was orally administered to male rats for six days. On day 8 (D8) after the first dose of FM, intratesticular testosterone (T) levels had dramatically increased, but daily sperm production on D36 was significantly decreased. The mRNA levels of testicular and pituitary genes on D8 were measured by semiquantitative RT-PCR. Among the six testicular steroidogenic enzyme genes, the mRNAs of the P450 side chain cleavage, P450 17α/C_{17-20} lyase, and 3β-hydroxysteroid dehydrogenase type I (3βHSD) genes significantly increased, whereas 17β-hydroxysteroid dehydrogenase type III slightly decreased. Among the three steroid receptors examined, androgen receptor (AR) and glucocorticoid receptor (GR) mRNAs were significantly down-regulated (29% and 35%, respectively) in the testis, but there was no change in estrogen receptor α. There were no clear changes in expression of the gonadotropin receptors and Sertoli cell specific genes, but a slight increase was observed in expression of the lactose dehydrogenase-c mRNA, a germ cell specific gene. Among the three immediate early genes, c-myc mRNA was increased approximately 1.4-fold. In the pituitary, on the other hand, mRNAs for LHβ and FSHβ subunits and gonadotropin releasing hormone receptor had increased significantly. These results show that subacute FM administration first affected hypothalamus/pituitary hormone gene expression, then altered gonadotropin secretion, and subsequently induced over-expression of testicular steroidogenic enzyme genes. However, the significant up-regulation of 3βHSD and down-regulation of AR mRNAs, despite the higher level of intratesticular T, might be explained by an antagonistic action of hydroxyflutamide retained in the testis. The profiles of alterations in gene expression observed will provide important information for the screening of adult male animals for anti-androgenic chemicals.

Key words: Antiandrogen, Flutamide, Testis, Pituitary, Androgen receptor

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The anti-androgen, flutamide (FM), is a non-steroidal androgen receptor (AR) antagonist, that interferes with endogenous androgen binding to ARs in target organs [1]. Several experiments in intact male rats have shown that FM increases

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serum gonadotropin and testosterone (T) concentrations and intratesticular T levels shortly after acute administration [2–4], and a 4-fold increase in serum T concentration was reported in male hamsters [5]. FM appears to first block the negative feedback of T in the hypothalamus and pituitary, and increased LH secretion then enhances T production in the testis [6]. FM administration has also been found to affect the initial step of spermatogenesis and cause a reduced sperm count due to inhibition of differentiation of spermatogonia to spermatocytes [4, 6, 7]. However, the mechanism by which FM inhibits spermatogenesis is still unclear.

To screen for and assess the health risks of exogenous endocrine-active chemicals (EACs), a 'Tiered-testing scheme' has been proposed [8, 9]. The Hershberger assay is generally used as an *in vivo* Tier I screening battery to screen for anti-androgenic EACs [10], but it requires that castration be performed, and the effects of test compounds are evaluated on the basis of accessory sex gland's weight alone. In addition to the Hershberger assay, a 15 days intact rat sub-chronic *in vivo* assay has been proposed [11]. This allows observation of the effects of test compounds in a relatively short time and a wide variety of organs, including the testis and pituitary. It permits evaluation of the effects of test compounds on the hypothalamo-pituitary-gonadal axis, which is the most important mechanism in the male reproductive system. However, the endpoints of this assay are still limited to only the weight data for the gonads and accessory sex glands and serum hormone levels (T, 17β-estradiol (E2), 5α-dihydrotestosterone (5αDHT), PRL, FSH, and LH) [12]. Although quantification of mRNA levels is still laborious, advances in biotechnology, especially in gene expression profiling systems, such as gene arrays or DNA chips, have made it easier and more convenient to obtain data on alterations of gene expressions in a variety of tissues exposed to test compounds. In terms of toxicogenomics [13, 14], it will be also necessary to provide precise information for databases concerning the alterations of gene expression when test compounds are administered to laboratory animals. Profiles of alterations of gene expression when an anti-androgen is administered to intact animals will be more important for researches of anti-androgenic EACs. However, precise information on alterations of gene expression, especially in the testes, is still limited for anti-androgenic compounds administered to intact laboratory animals.

In the present study, we analyzed the effects of subacute FM administration to adult male rats on the efficiency of spermatogenesis, serum and testicular hormone levels, and gene expression profiles for the establishment of a more reliable method of evaluating the effects of anti-androgenic compounds. The specific targets of this study were the alterations of gene expression in the testis and pituitary. Dramatic reductions in androgen and glucocorticoid receptor mRNA levels in the testis were found.

Materials and Methods

Materials

Flutamide (FM; 2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propamide) was purchased from Sigma (St. Louis, MO). The corn oil used to dissolve FM and used as the vehicle control was also bought from Sigma. Serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were determined with an enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Serum and intratesticular T levels were determined with an enzyme immunoassay (EIA) system (Cayman Chemical Co., Ann Arbor, MI). SuperScript™ II RNase H- Reverse Transcriptase and oligo(dT)12-18 primer were purchased from Life Technologies (Rockville, MD). LA Taq™ with 2 × GC Buffer I and Ex Taq™ with 10 × Ex Taq™ Buffer were bought from TaKaRa Biomedicals (Otsu, Shiga, Japan). The plasmid pGEM-T Easy vector was obtained from Promega Corp. (Madison, WI).

Animals and treatment

Male Holtzman rats were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and bred in our own facility. They were maintained in a controlled environment at a temperature of 24 ± 1 C, humidity of 45 ± 5%, and on a 12/12 h light/dark cycle. The animals were given access to food and distilled water *ad libitum*. At 13 weeks of age males were randomly selected and divided into four groups (n=8), so that average body weight was the same in each group. The rats were given

vehicle (corn oil) or FM (25 mg/kg/day, in corn oil) orally for 6 days. At 8 days (D8) and 36 days (D36) after the first dose, rats were sacrificed by exsanguination from the aorta under ether anesthesia. The serum was separated and stored at –20 C in a freezer. Both testes were excised, and the surrounding adipose tissue was carefully removed. The pituitary was also quickly removed. The specimens were frozen in liquid nitrogen immediately after excision, and maintained at –80 C until daily sperm production and intratesticular T concentration were measured and RNA was extracted.

Daily sperm production

Testes were homogenized in 10 mM phosphate-buffered saline (PBS, pH 7.4) in a polytron homogenizer, and the homogenization-resistant spermatid nuclei were counted with a hemocytometer. The numbers of homogenization-resistant spermatid nuclei per testis were calculated and then divided by 6.1 days to convert them to testicular daily sperm production (DSP) values [15].

Hormone assay

Serum LH, FSH, and T and intratesticular T levels were determined by using the corresponding EIA systems. The serum samples collected were directly applied to the well in the kit, and measurements were made according to the procedure described by the manufacturer. To measure intratesticular T levels, the frozen right testis was homogenized in 8 ml of 10 mM PBS with a polytron homogenizer. The homogenate (2 ml) was then extracted with diethylether, and the ether phase was dried in air. The serum was also extracted with diethylether, and the ether phase was dried in air. The dried lipophilic substances were resuspended with the proper volume of the buffer in the kit, and the measurements were made according to the procedure described by the manufacturer.

Semiquantitative RT-PCR

Total RNA was extracted from the testes and pituitaries (n = 8) by the basic protocol of Chomczynski and Sacchi [16]. RNA samples (testis, 8 μg; pituitary, 1.25 μg) were reverse-transcribed for 50 min at 42 C in a 40-μl reaction mixture with 400 units of SuperScript™ II reverse transcriptase and 1 μg of oligo(dT)12–18 primer according to the standard protocol of the supplier. Each PCR reaction mixture (50 μl) contained 0.2 mM of each dNTP mixture, 0.4 μM of each primer, and 1 μl of the cDNA reverse-transcribed as described above. Taq polymerase was added to the reaction tube as 2.5 units of TaKaRa LA Taq™ polymerase in 1 × GC Buffer I or 1.25 units of TaKaRa Ex Taq™ polymerase in 1 × Ex Taq™ Buffer. PCR was performed by denaturing at 94 C for 30 sec, annealing for 30 sec, and extension at 72 C for 45 sec. Table 1 shows the primer sequences, PCR-product sizes, optimized cycles, annealing temperatures, Taq polymerase used, and GenBank accession numbers for all genes examined in this study: the genes coding cytochrome P450 side chain cleavage (P450scc), cytochrome P450 17α/C_{17-20} lyase (P450c17), 3β-hydroxysteroid dehydrogenase type I (3βHSD), 17β-hydroxysteroid dehydrogenase type III (17βHSD), cytochrome P450 aromatase (P450arom), 5α-reductase type I (5αR-I), androgen receptor (AR), estrogen receptor α (ERα), glucocorticoid receptor (GR), LH receptor (LHR), FSH receptor (FSHR), protamine-2 (Pm2), lactose dehydrogenase-c (LDH-C), acrosin, low affinity nerve growth factor receptor (LNGFR), androgen binding protein (ABP), transferrin (Tf), Sertoli glycoprotein-1 (SGP1), α subunit of LH and FSH (Gnα), β subunit of LH (LHβ), β subunit of FSH (FSHβ), gonadotropin releasing hormone receptor (GnRHR), β subunit of thyrotropin stimulating hormone (TSHβ), prolactin (PRL), c-fos, c-jun, c-myc, Egr1, cyclophilin (CP), and β-actin. The PCR products (5 μl) were then separated on 2% agarose gel. The bands on the UV-transilluminated gel were converted into digital images with a gel analyzer (ATTO Inc., Bunkyo-ku, Tokyo, Japan), and the amounts of RT-PCR products were quantified with Scion Images software (Scion Corporation, Frederick, MD). PCR products for CP or β-actin were used as an internal standard. To determine the sequences, the PCR product for each gene was subcloned into pGEM-T Easy vectors and sequenced by the dideoxynucleotide chain termination method using the ABI Prism BigDye terminator cycle sequencing kit (PE-Biosystems, Foster City, CA).

Statistical analysis

StatView for Windows version 5.0 (SAS Institute Inc., Cary, NC) was used for the statistical analysis.

Table 1. Primers used for semiquantitative RT-PCR

Gene	Primer sequence		Product size (bp)	Cycle used	Anneal Temp (C)	Taq used	GenBank accession no.
	Forward primer (5' to 3')	Reverse primer (5' to 3')					
P450scc	CGCTCAGTGCTGGTCAAAA	TCTGGTAGACGGCGTCGAT	688	23	55	Ex	J05156
P450c17	GACCAAGGGAAAGGCGT	GCATCCACGATACCCTC	302	24	55	Ex	M22204
3βHSD	TTGGTGCAGGAGAAAGAAC	CCGCAAGTATCATGACAGA	547	24	55	Ex	M38178
17βHSD	TTCTGCAAGGCTTTACCAGG	ACAAACTCATCGGCGGTCTT	653	26	55	Ex	AF035156
P450arom	GTGCCTGCAACTACTACAATAAG	CTCATACTTTCTGTAGAGCCAAG	521	32	60	LA	M33986
5αR-I	ATGGAGTTGGATGAGCTGTG	TCCTCAGGATGTGGTCTGAG	508	25	60	LA	J05035
AR	TGCTGCCTTGTTATCTAGTCTCA	ACCATATGGGACTTGATTAGCAG	570	26	60	Ex	M20133
ERα	TTACGAAGTGGGCATGATGA	ATCTTGTCCAGGACTCGGTG	711	26	60	Ex	Y00102
GR	GGAGAATTATGACCACACTCAAC	GCAGTAGGTAAGGAGATTCTCAA	539	26	60	Ex	M14053
LHR	CTCACCTATCTCCCTGTCAAAGT	ACAGACTCGTTATTCATCCCTTG	365	25	60	Ex	M63919,M63922
FSHR	GGTTTCTTATGTGTAACCTCGCC	AACTATTGGTGACTCTGGGAGCT	852	26	60	LA	L02842
Pm2	GGTTCGCTACCGAATGA	CTTCGGGATCTTCTGCA	265	25	55	Ex	X14674
LDH-C	CAAGGAGCAGCTAATTCAGAACC	CTTCTCTCCAATCAGGTAACGGA	520	22	60	LA	NM017266
Acrosin	TCACGGCTGCTCACTGCTTCGAT	CGACGCGTGTCTCTGCACATGAG	502	25	60	LA	X59254
LNGFR	CAACCCTTGTGCAGATGAACTGT	CTAAATAGAGGGGGTGCCCACTC	732	27	60	LA	X05137
ABP	CTATGCCTGAGACAAGTTTCTGC	GTAGAAAGGACCTCCATCTTTGG	533	21	60	Ex	M19993
Tf	AACCCACAATCAGATGTCTTTCC	TGGAAGTCTTCCTGCTTCAGATC	428	22	60	Ex	D38380
SGP1	GTTCTGCCAACCCGAATTTGGTG	CAATGATCGACAGCATTGCATCG	484	20	60	Ex	M19936
Gnα	ATGGATTGCTACAGAAGAT	TGAAGTATAAGGGATGTGA	501	19	60	Ex	V01252
LHβ	CTGGCTGCAGAGAATGAGT	GAAGGTCACAGGTCATTGG	292	20	60	Ex	J00749
FSHβ	TGTACGAGACCATAAGATTG	TTGAGTATCCTAACCTTGTG	757	22	60	Ex	D00577
GnRHR	ATGGCTAACAATGCGTC	ATATAACTGTGGTCCCG	877	26	50	LA	L25053
TSHβ	ATGAATGCTGTCGTTCTCT	CAGCCTCGTGTGTACAGTC	358	20	60	Ex	K01935
PRL	ACACTCCTCCTGCTGATGATGTC	GACAATTTGGCACCTCAGGAACT	630	17	60	Ex	NM012629
c-fos	GAGATTGCCAATCTACTGAA	AGACAAAGGAAGACGTATAG	484	31[a],27[b]	58	Ex	X06769
c-jun	GATCGCCCGGCTAGAGGAAAAAG	TCGCAACCCAGTCCATCTTGTGT	306	31[a],28[b]	65	LA	X17163
c-myc	CACCAGCAGCGACTCTGAAGAAG	ACTTCCGGTCAGTTTATGCACCA	593	31[a],29[b]	65	LA	NM012603
Egr1	CCATCACCTATACTGGCCGCTTC	GGGTTTGATGAGTTGGGACTGGT	569	30	65	LA	NM012551
CP	TCTGAGCACTGGGGAGAAAG	AGGGGAATGAGGAAAATATGG	524	18[a],21[b]	60	Ex	M19533
β-actin	CTGTGCCCATCTATGAGGGTTAC	AATCCACACAGAGTACTTGCGCT	359	22	60	Ex	V01217

[a] used for the testis samples.
[b] used for the pituitary samples.

All results are shown as means ± SE. Data were analyzed by the two-tailed Student's *t*-test. Significance was accepted at $p<0.05$.

Results

Testicular weight and daily sperm production

Table 2 shows the body weights, testicular weights, daily sperm production (DSP), and efficiency of testicular sperm production calculated by dividing the DSP number by testicular weight (DSP/gt). Administration of FM for 6 days did not result in any changes in body weight on either D8 or D36 compared to the vehicle-treated control. The testicular weight of the FM-treated animals on D8 was significantly higher than in the control group, but there was no difference on D36. DSP and DSP/gt, on the other hand, were significantly lower on D36 (79.5% and 76.9% of the control, respectively), but there was no difference on D8.

Serum and testicular hormone concentrations

Table 3 shows the changes in serum LH, FSH, and T levels. The LH level was significantly increased (1.2-fold) by FM on D8, but there was no change on D36. The FSH levels on both D8 and D36 were slightly higher than in the control, but the difference was not statistically significant. Serum T was increased 3.4-fold over control on D8 ($p<0.001$), but there was no significant difference on D36. Similarly, there was a 4-fold increase in intratesticular T level on D8 (FM, 720 ng/g testis; control, 170 ng/g testis; Fig. 1). However, no difference between FM-treated and vehicle-treated animals was observed in intratesticular T levels on D36, which was slightly lower than that of control (Fig. 1).

Table 2. Effects of subacute FM administration on the reproductive system of the adult male rat

	Control	FM
D8		
Body weight (g)	491.8 ± 11.9	470.9 ± 11.5
Paired testicular weight (g)	3.71 ± 0.07	3.95 ± 0.09*
DSP (× 10^6)	4.20 ± 0.01	4.41 ± 0.12
DSP/gt (× 10^6)	2.27 ± 0.07	2.23 ± 0.07
D36		
Body weight (g)	518.4 ± 19.3	528.3 ± 12.8
Paired testicular weight (g)	3.82 ± 0.08	3.78 ± 0.27
DSP (× 10^6)	4.10 ± 0.11	3.26 ± 0.10**
DSP/gt (× 10^6)	2.12 ± 0.05	1.63 ± 0.04**

Male rats 13 weeks old were daily dosed vehicle or FM (25 mg/kg) for six days. Data were expressed as means ± SE (n= 8; * $p<0.01$; ** $p<0.001$).

Table 3. Effects of subacute FM administration on the serum hormone levels of the adult male rat

	Control	FM
D8		
LH (ng/ml)	19.21 ± 0.34	23.62 ± 0.28**
FSH (ng/ml)	129.02 ± 13.50	137.88 ± 7.25
Testosteorne (ng/ml)	1.40 ± 0.78	4.73 ± 0.59**
D36		
LH (ng/ml)	22.56 ± 1.31	22.25 ± 2.40
FSH (ng/ml)	88.69 ± 10.51	109.37 ± 10.45
Testosteorne (ng/ml)	0.42 ± 0.20	0.68 ± 0.15

Male rats 13 weeks old were daily dosed vehicle or FM (25 mg/kg) for six days. Data were expressed as means ± SE (n= 8; ** $p<0.01$).

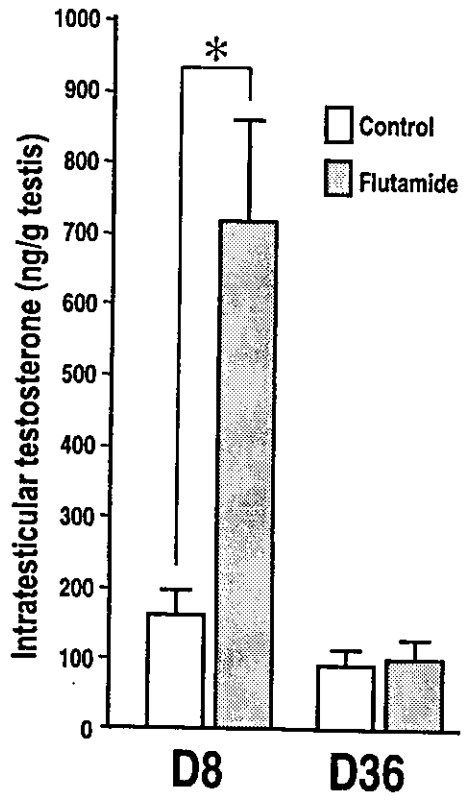


Fig. 1. Effect of subacute FM administration on the intratesticular testosterone concentration in male rats. Intratesticular T levels were determined as described in Materials and Methods. The values are expressed as means ± SE (n=8). A significant increase ($P<0.001$) in intratesticular T level was observed on D8 in FM treated groups.

Gene expression profiles

Since the samples on D8 were expected to show more drastic changes by FM than on D36, total RNAs from testis and pituitary on D8 were subjected to semiquantitative RT-PCR. Figure 2 shows the amplification curves of all genes analyzed in this study to optimize the cycles for quantification. In the testis sample from a control rat, the PCR product for cyclophilin (CP) mRNA was first detected in the early cycles, suggesting that CP was the most abundant gene in the testis. The PCR product for P450arom mRNA, on the other hand, could not be detected at less than 30 cycles, suggesting an extremely low level in the testis. Actually, we used two sets of primers for AR with DNA sequences located in different regions in the AR open reading frame. The band intensities of the two PCR products of AR were the same on the corresponding cycles (data not shown).

Steroidogenic enzymes: Among the six steroidogenic enzymes examined, marked up-regulations, as large as 2-fold, of P450scc and P450c17 mRNAs were detected (Fig. 3). When standardized by CP, the 3βHSD mRNA level was 2.4-fold higher than the control (Fig. 3). 17βHSD mRNA was slightly reduced by FM (statistically significant when corrected by CP, but not by β-actin). The 5αR-I mRNA level was reduced to 79% (by CP), but no significant change was observed in P450arom (Fig. 3). Other steroidogenic enzyme genes, 17β-HSD-type I, 17β-HSD-type II, and 5α-reductase type II, were examined in testis samples, but no signals were detected even after 40 cycles of the PCR reaction (data not shown).

Steroid hormone receptors: In the testis, significant decreases in the mRNAs of two of the

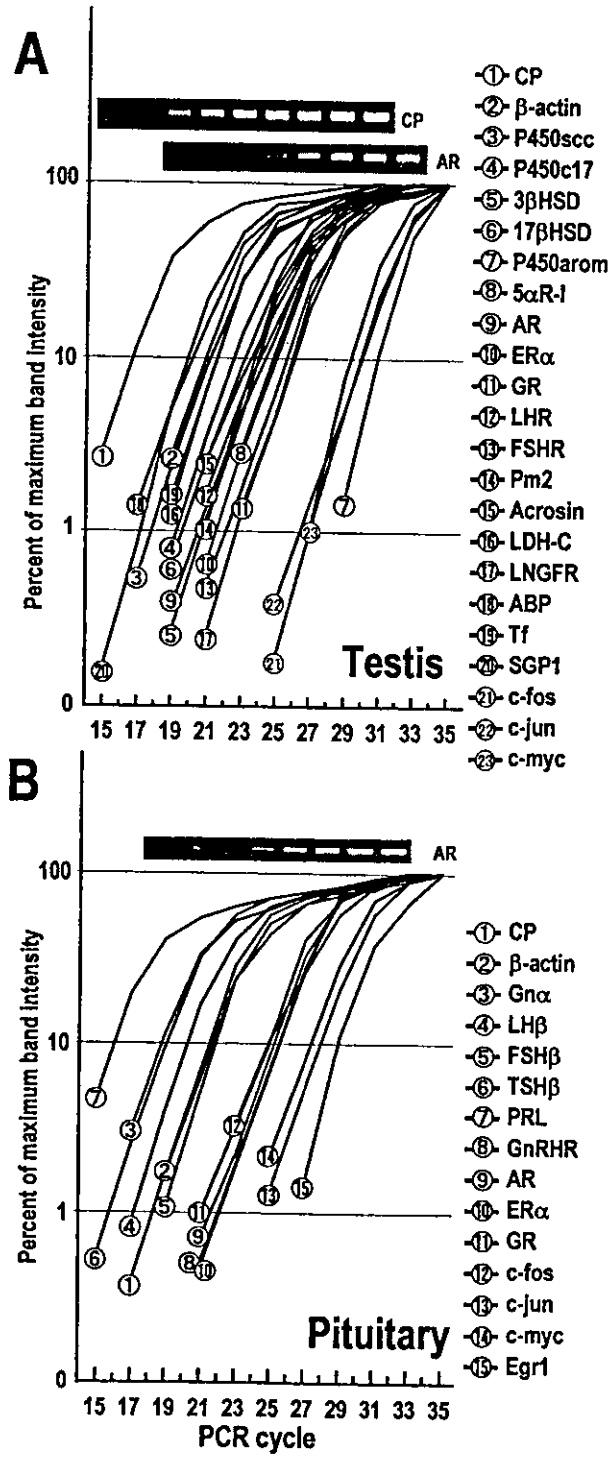


Fig. 2. Optimization of amplification cycles for each gene in the semiquantitative RT-PCR analysis. A, Amplification curves of PCR-products of 23 genes from the testicular cDNA sample of a vehicle-treated rat. B, Amplification curves of PCR-products of 15 genes in the pituitary. The PCR cycles used for semiquantitative analysis were determined from the linear regression phase of these curves and are indicated in Table 1.

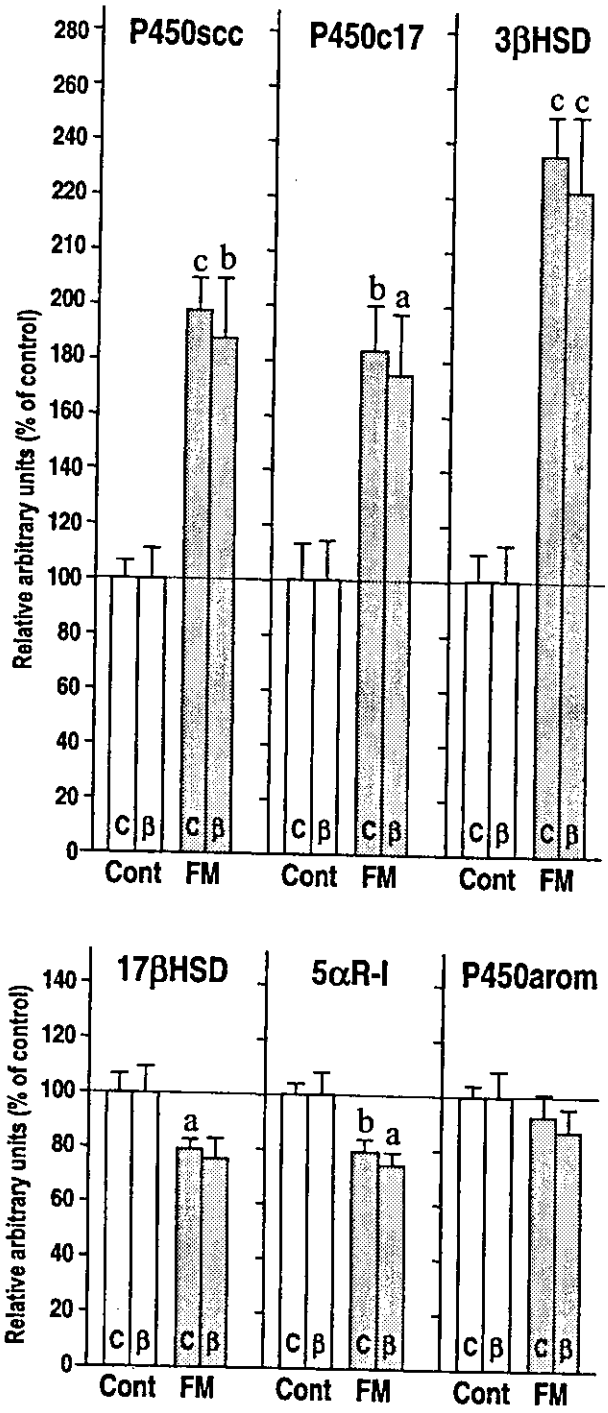


Fig. 3. Semiquantitative RT-PCR analysis of the effect of FM on testicular steroidogenic enzyme mRNA levels. Relative amounts of RT-PCR products from 6 steroidogenic enzyme mRNAs (P450scc, P450c17, 3βHDS, 17βHSD, 5αR-I, P450arom) were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n=8). Note the significant increases of P450scc, P450c17, and 3βHSD mRNA levels. Significant differences from means in the control were analyzed with Student's *t*-test (a: P<0.05, b: P<0.01, c: P<0.001).

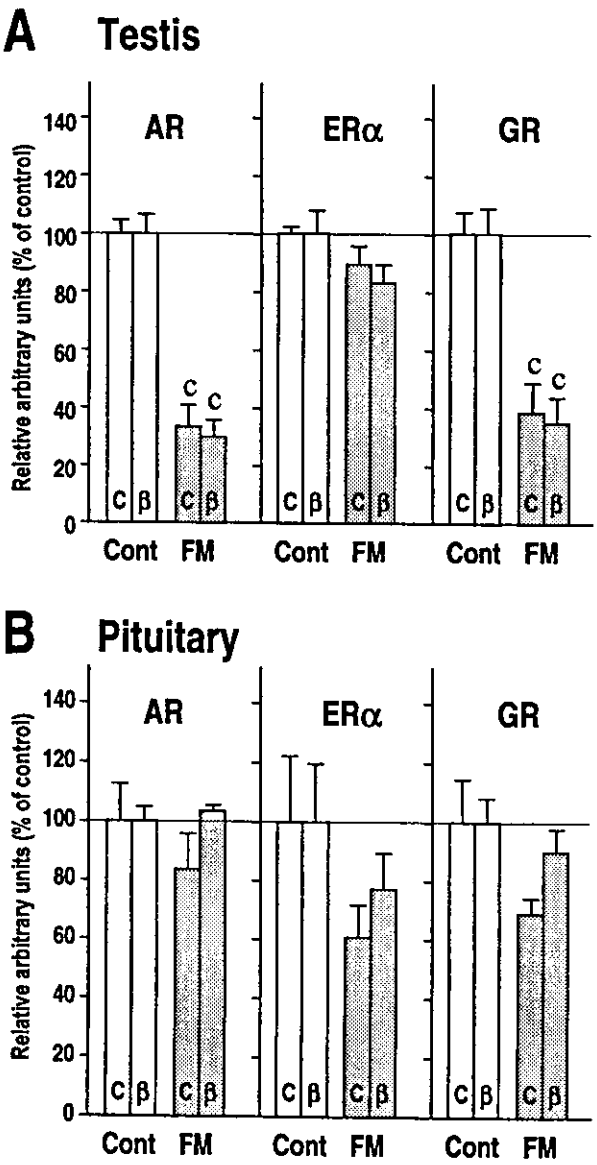


Fig. 4. Semiquantitative RT-PCR analysis of the effect of FM on the mRNA levels of testicular (A) and pituitary (B) steroid hormone receptor genes. Relative amounts of RT-PCR products of the steroid hormone receptor genes (AR, ERα, GR) were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n = 8). Note the significant decreases in AR and GR mRNA levels in the testis. Differences between the means and the control were analyzed for significance with Student's *t*-test (a: P<0.05, c: P<0.001).

three steroid receptors, AR and GR, were found (29% and 35%, respectively, of the control by β-actin standardization), but there was no significant change in ERα mRNA (Fig. 4A). There were no such decreases in AR or GR mRNA levels in the pituitary from the same individuals on D8 (Fig. 4B). The ERα level in the pituitary was somewhat decreased, but the difference was not statistically significant. Other steroid receptor genes, the genes for ERβ and progesterone receptor, were examined in the testis samples, but no signals were detected even at 40 cycles of the PCR reaction (data not shown).

Gonadotropin receptors, Sertoli cell proteins and spermatogenic cell-specific genes: The levels of gonadotropin receptors, LHR and FSHR, expressed in Leydig cells and Sertoli cells, respectively, were somewhat lower than in the control, but the differences were not statistically significant (Fig. 5). LNGFR, also expressed in Sertoli cells, was unchanged (Fig. 5). Although a slight but not statistically significant increase was seen in ABP, transferrin and SGP1, both major secretory proteins of Sertoli cells, were unaltered by FM administration. The mRNAs of germ cell specific genes, i.e., the gene for protamine-2, specifically expressed in haploid germ cells, and the gene for LDH-C, which is transiently expressed in spermatocytes and early phase spermatids, were slightly increased, but statistical significance was only detected for LDH-C mRNA (Fig. 5). The mRNA of acrosin, a pachytene stage specific protein, was completely unchanged by FM.

Gonadotropin subunits and other pituitary genes: Among the three genes for gonadotropin subunits expressed in the pituitary, significantly increased mRNA levels of LHβ and FSHβ subunit were detected (2.9-fold and 2.2-fold, respectively, the control levels by β-actin-standardization), and a 1.5-fold increase in LH/FSHα (Gnα) subunit mRNA was seen, but the difference was not statistically significant (Fig. 6). GnRHR mRNA increased significantly (2.1-fold the control value by β-actin-standardization), but there were no changes in TSHβ subunit or PRL mRNA levels.

Immediate early genes: No significant increases in the mRNAs of the immediate early genes c-fos and c-jun, were detected in the testis, but a 1.4-fold increase in c-myc mRNA (by CP-standardization) was observed (Fig. 7A). In the pituitary, c-fos, c-jun, and Egr1 mRNAs had decreased to 74%, 67%, and 77%, respectively, of control levels (by CP-standardization), but only the difference in c-jun mRNA was statistically significant (Fig. 7B).

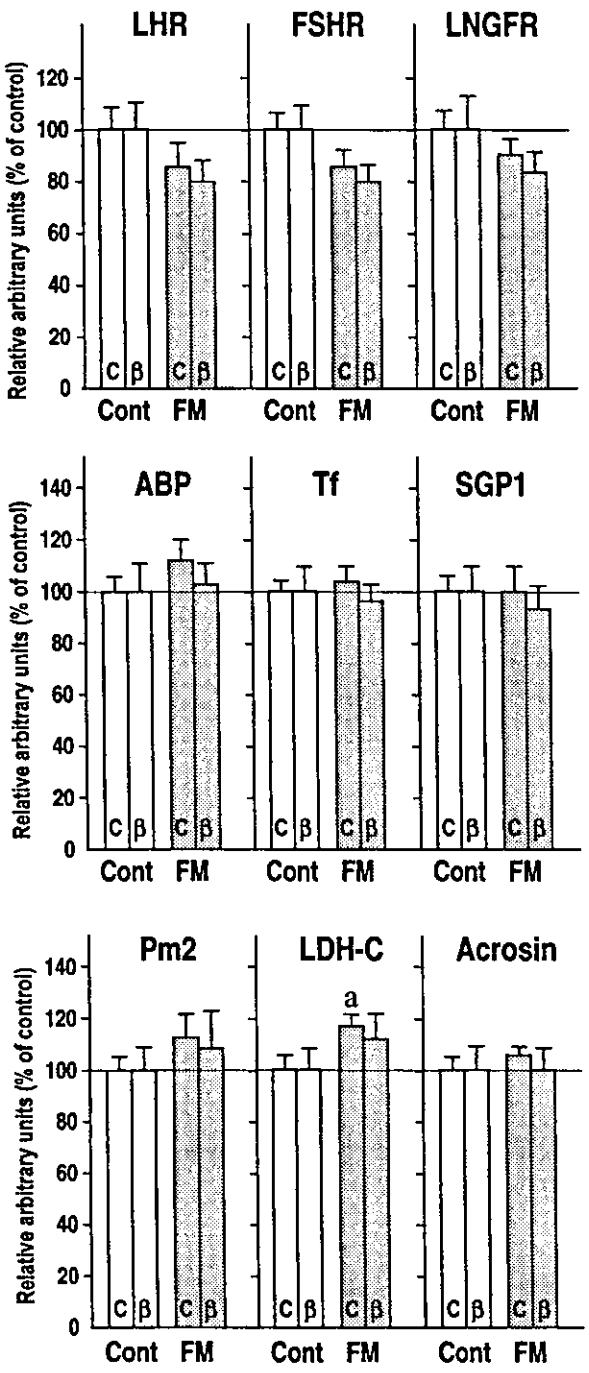


Fig. 5. Semiquantitative RT-PCR analysis of the effect of FM on testicular gonadotropin receptor and Sertoli cell and spermatogenic cell specific gene mRNA levels. Relative amounts of RT-PCR products from gonadotropin receptor mRNAs (LHR, FSHR), genes expressed by Sertoli cells (LNGFR, ABP, Tf, SGP-1), and spermatogenic cell specific genes (Pm2, LDH-C, acrosin) were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n=8). Differences between the means and the control were analyzed for significance with Student's *t*-test (a: $P<0.05$).

Discussion

Subacute FM administration affects the early stage of rat spermatogenesis

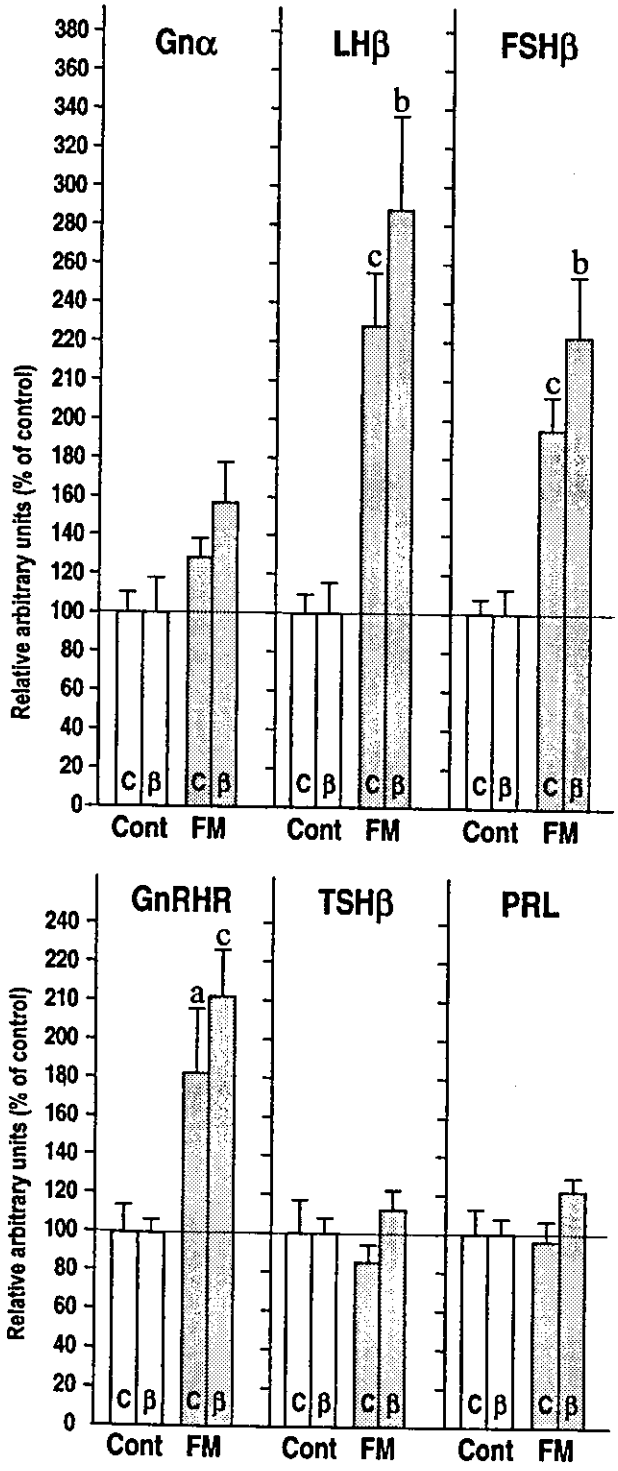
The present study showed a slight but statistically significant increase in testicular weight in the FM-treated rats on D8, but no changes in daily sperm production (DSP). By contrast, on D36, no change in testicular weight was detected, but DSP was significantly reduced. Since there was no change in DSP, the increase in testicular weight appeared to be attributable to an increase in volume of other compartments, not the homogenization-resistant spermatid heads. The semiquantitative RT-PCR analysis revealed slight increases in the mRNA levels (not significant) of ABP, a Sertoli cell-secreted protein [17] and protamine-2, a haploid germ cell specific protein [18], and a significant increase in the mRNA level of LDH-C, a germ cell specific glycolytic enzyme [19]. The increase in testicular weight on D8 may be explained by the increased levels of these relatively abundant testicular proteins. Actually their genes should have been stimulated by the increased level of gonadotropins or T, as described below.

It has been well documented that it takes about 49 days for type A spermatogonia to develop to step 19 spermatids, which are homogenization-resistant, in the rat seminiferous tubule [20]. Thus, the reduction of DSP on D36, but not on D8, means that FM administration suppressed the early stage of spermatogenesis, that is, differentiation of spermatogonia or early phase spermatocytes, and the effect appears to be transient. Our method, i.e., measuring DSP 36 days after the first dose of a test compound, would be useful for detecting slight changes in numbers of elongated spermatids, if the test compounds affected the proliferation and differentiation of spermatogonia or spermatocytes [21]. Consistent with our data, early histopathological studies in male rats given FM for 15 days clearly demonstrated that FM affected spermatogenesis: the ratio of spermatogonia to spermatids was reduced on the day of the final injection, indicating that FM affected the early stages of spermatogenesis [4, 6, 7].

FM administration blocks negative feedback of T to the hypothalamus and pituitary

The dramatic increase in serum and intratesticular T levels shortly after FM

administration (on D8) must have been due to an increase in LH secretion, and this was corroborated by the increased level of serum LH and pituitary LHβ mRNA levels. It can be explained by an inhibitory action of FM on the negative feedback effects of T on the hypothalamus and pituitary, and subsequent enhancement of the LH or GnRH pulse [6, 7]. In the present study, there was no significant increase in LH/FSHα (Gnα) subunit mRNA. Gnα expression has been documented to also be regulated by GnRH pulse, because it has been reported to increase 3-fold in male rats one week after castration [22, 23]. FM administration also significantly increased the GnRHR mRNA level (2.1-fold) in the pituitary (Fig. 6). In the male rats, GnRHR mRNA has been reported to increase 5-fold in male rats 21 days after castration [24, 25], and this increase presumably is due to an increased GnRH pulse caused by termination of the negative feedback effect of T. Thus, the above result also supports the contention that FM administration inhibits the negative feedback effects of T on the hypothalamus and pituitary enhancing the GnRH pulse.



However, Dalkin and coworkers [22, 23] reported that 6-fold increases in LHβ mRNA and 4-fold increases in FSHβ mRNA were observed in castrated rats stimulated by exogenous GnRH pulses with an implanted silastic tube containing T, whereas the increase in Gnα was not statistically significant. We detected a 1.5-fold increase in Gnα mRNA level, although the increase was not statistically significant, which seems similar to the findings in their report, suggesting that endogenous T was still effective in the pituitary of rats shortly after FM administration. In addition, there was no significant change in serum FSH level, whereas the FSHβ mRNA content in the pituitary was 2.2-fold higher than in the control (Fig. 6). This difference might be due to the sensitivity of the FSH assay system. However, we did not detect any significant increase in mRNA levels of testicular ABP or Tf, the Sertoli cell secretory proteins, which are well known to be up-regulated by FSH

Fig. 6. Semiquantitative RT-PCR analysis of the effect of FM on pituitary hormone and receptor gene mRNA levels. Relative amounts of RT-PCR products from gonadotropin subunit (Gnα LHβ, FSHβ), gonadotropin releasing hormone receptor (GnRHR), and other hormone (TSHβ, PRL) mRNAs were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n=8). Note the significant increases in LHβ and FSHβ subunit mRNA levels. Differences between the means and the control were analyzed for significance with Student's *t*-test (a: $P<0.05$, b: $P<0.01$, c: $P<0.001$).

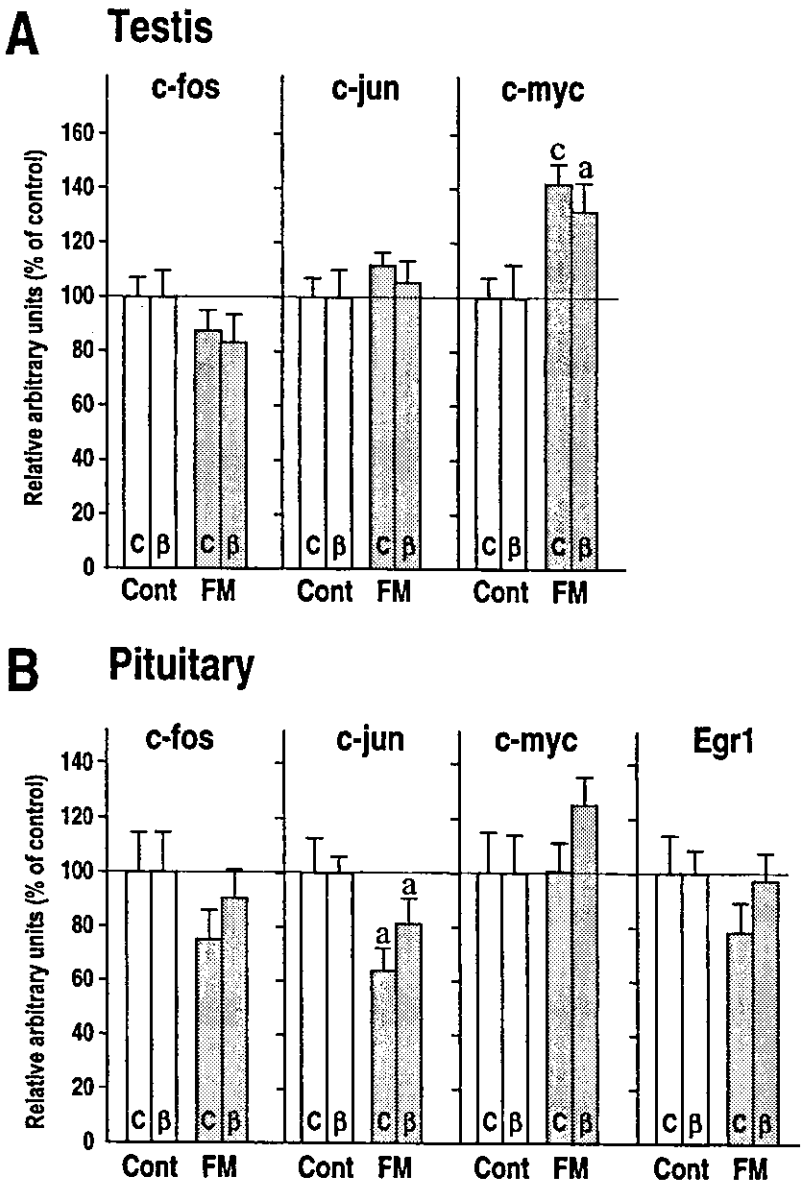


Fig. 7. Semiquantitative RT-PCR analysis of the effect of FM on testicular (A) and pituitary (B) immediate early gene mRNA levels. Relative amounts of RT-PCR products of immediate early genes (c-myc, c-fos, c-jun, Egr1) were calculated by dividing by one of the two internal controls, CP (C) or β-actin (β). The values expressed are means ± SE (n=8). Note the significant increase in c-myc mRNA levels in the testis. Differences between the means and the control were analyzed for significance with Student's *t*-test (a: $P<0.05$, b: $P<0.01$, c: $P<0.001$).

stimulation [26]. Testicular and epididymal ABP and plasma Tf were found to be up-regulated in hamsters and humans after FM administration, and the FSH level increased simultaneously [5, 27]. These findings strongly suggest that the serum FSH level on D8 (2 days after the final dose) was already reduced in our study. There is a report, on the other hand, that T increases the FSHβ mRNA content in the pituitary selectively by post-transcriptional mechanisms, independent of the GnRH action, based on the result that the addition of T to rats castrated and implanted with GnRH