Lack of Modification by Environmental Estrogenic Compounds of Thyroid Carcinogenesis in Ovariectomized Rats Pretreated with N-bis(2-hydroxypropyl)nitrosamine (DHPN)

Hwa-Young Son,1, 3 Akiyoshi Nishikawa,1 Takako Ikeda,1, 2 Fumio Furukawa1 and Masao Hirose1
1Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501 and 2Showa Women’s University, 1-7 Taishido, Setagaya-ku, Tokyo 154-8533

The effects of environmental estrogenic compounds, soy isoflavone mixture (SI), genistein (GEN), and nonylphenol (NP), and the possible goitrogen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), on thyroid carcinogenesis were investigated in ovariectomized (OVX) female rats. Five-week-old OVX F344 rats were given a single subcutaneous injection of N-bis(2-hydroxypropyl)nitrosamine (DHPN; 2400 mg/kg, body weight) or vehicle alone. Starting 1 week later, GEN (250 or 25 ppm in diet), SI (400 ppm in diet), NP (250 or 25 ppm in diet), MX (30 ppm, in drinking water), sulfadimethoxine (SDM), a known thyroid tumor-promoter (1000 ppm in drinking water), or β-estradiol 3-benzoate (EB), a synthetic estrogen (0.5 mg in cholesterol pellet, s.c.) were administered for 12 weeks. SDM and EB were included as positive controls. At sacrifice the major organs including the thyroid, pituitary, liver, kidney, uterus, vagina, brain and pancreas were collected and histopathological observation was performed. Thyroid weights were significantly increased (P<0.001) only in the SDM treatment group and pituitary weights were elevated with SDM (P<0.05) and EB (P<0.001), Kidney and uterine weights were also significantly increased (P<0.05) by EB. Histopathologically, proliferative lesions of the thyroid were only observed in the SDM treatment group and of the pituitary in the SDM or EB treatment groups. Renal tubule lesions, uterine squamous metaplasia, vaginal keratinization and telangiectasia of pancreatic islets were also observed with EB. There were no organ weight changes or histopathological lesions in the major organs, including the thyroid, in the GEN, SI, MX or NP treatment groups. Our results thus indicated a lack of modifying effects on thyroid carcinogenesis in female OVX rats, in agreement with our previous finding in males.

Key words: Thyroid carcinogenesis — Ovariectomy — Genistein — Nonylphenol — 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone

Considerable attention has recently been focused on environmental estrogenic chemicals (EECs) that can disrupt the endocrine system. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) under the US Environmental Protection Agency (EPA) has recommended screening of compounds for their potential to act as estrogen receptor (ER) or androgen receptor (AR) agonists or antagonists, or for their ability to alter thyroid function.1) Endocrine disrupting chemicals (EDCs) may interfere with thyroid hormone function and homeostasis by inhibiting synthesis, altering serum transport proteins, or increasing catabolism of thyroid hormones. There are two basic mechanisms whereby chemicals induce thyroid gland tumors in rodents. One involves direct carcinogenic effects at the DNA level in follicular cells. The other is associated with hypersecretion of thyroid stimulating hormone (TSH) that stimulates growth of follicular epithelium, resulting in thyroid neoplasias.2) Recently, receptor binding activity has been noted for EDCs like the ER agonists bisphenol A and methoxychlor and the AR antagonists linuron and p,p'-DDE.3) However, the actual impact of such compounds on the thyroid gland has not been well defined. Castration markedly reduces radiation-induced thyroid carcinomas4) and testosterone causes elevation of both baseline and TSH-releasing hormone (TRH)-stimulated TSH levels,5) and increase of methylthiouracil or radiation-induced rat thyroid tumors.4, 6) Furthermore, ovariectomy (OVX) was found to lower the incidence of thyroid carcinomas in female Long-Evans rats,7) pointing to roles for both sex hormones in the development of thyroid cancer.5) For accessing the impact of estrogenicity of estrogenic chemicals, immature or OVX females are necessary, since they are much more sensitive to estrogenic chemicals than intact mature females.

In the present study, the modifying effects of the EECs, soy isoflavone mixture (SI), genistein (GEN) and non-
ylphenol (NP) on thyroid carcinogenesis were investigated in OVX rats pretreated with N-bis(2-hydroxypropyl)nitro-

Materials and Methods

Animals Specific-pathogen-free OVX F344 rats, 4 weeks old, were obtained from Japan SL C Inc. (Shizuoka) and housed five to a polycarbonate cage with wood chips as bedding in an air-conditioned animal room (room temperature, 23±2°C; relative humidity, 60±5%; a 12h light/dark cycle). Animals without any abnormal findings after a 1-week acclimation period were selected for the present study. Powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) from which soy constituents were eliminated and ion exchange water as the drinking water ad libitum were used throughout the study.

Chemicals DHPN was obtained from Nacalai Tesque Inc. (Kyoto) and SDM from Sigma Chemicals (St. Louis, MO). MX was synthesized from tetrachloroacetone and (carbomethoxylene) triphenylphosphorane according to the method of Padmapria et al. Its purity was determined as 97% by high-performance liquid chromatography (R-ODS-5 column, YNS, Osaka). NP and SI were respectively obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and Kikkoman Co., Ltd. (Tokyo). SI contained more than 30% isoflavone aglycone (genistin 12–18%, diadzein 12–18% and glycitein 2–4%). GEN was synthesized as described previously and its purity proved to be higher than 97%. EB and cholesterol were obtained from Wako Pure Chemical Industries Ltd. (Osaka). EB-containing pellets were prepared in medical grade Silastic tubes (Kaneka Medix, Co., Osaka), filled with 33 mg of EB mixed with 1 g of cholesterol and 1 ml of olive oil. The content of EB in each pellet (1 cm) was approx. 0.5 mg.

Dose selection for each compound was based on previous reports of thyrotropic, carcinogenic or endocrine-modulating effects. Because 1000 ppm of SDM induced thyroid proliferative lesions within 12 weeks, we selected the SDM treatment group as a positive control. The dose (400 ppm) of SI used in this study is as high as that contained in 20% defatted soybeans, which induced thyroid proliferative lesions. Further, 250 ppm of GEN is approximately the concentration present in ~20% soybean protein diet, and exhibits antioxidant activities. The dose of 250 ppm of NP administered in the diet corresponds to approximately 11–44 mg/kg/day in rats and approximately 1/10 of the maximum tolerated dose (MTD). The minimum detection level for estrogenic response to NP in rodents, following oral administration, was ca. 40 mg/kg/day. The dose (30 ppm) of MX selected in this study induced cell proliferation as well as lipid peroxidation in the gastric mucosa and exerted promoting effects in two-stage glandular stomach carcinogenesis in rats.

Experimental procedures Rats were divided into 10 groups, each consisting of 10 animals, so that initial mean body weights of each group were similar. Rats of 9 groups received a single s.c. injection of 2400 mg/kg DHPN. From 1 week thereafter, they were given no further treatment, SDM (1000 ppm in drinking water), SI (400 ppm in diet), GEN (250 or 25 ppm in diet), NP (250 or 25 ppm in diet), MX (30 ppm in drinking water) or EB (0.5 mg) for 12 weeks. EB pellets were implanted in the subcutis of the interscapular area under light anesthesia by ethyl ether and replaced every 4 weeks. Non-treatment control rats were fed basal diet without DHPN pretreatment for up to 12 weeks. The experimental diets were prepared in Oriental Yeast Co. (Tokyo) by adding test chemicals to the soy protein-free CRF-1 diet. At autopsy, major organs including the thyroid, pituitary, liver, kidney and uterus were carefully examined macroscopically, and then weighed. In addition to the organs mentioned above, the brain, vagina and pancreas were fixed in 10% phosphate-buffered formalin, and sections stained with hematoxylin and eosin were observed under a microscope. The results were statistically analyzed using ANOVA followed by Student’s t test. Differences were considered statistically significant at P<0.05.

Results

Test chemical intakes Daily intakes of test chemicals estimated from food and water consumption data are shown in Tables I and II. The intakes of test chemicals were well correlated with the doses applied.

Body and organ weights Data for mean body weight gain are illustrated in Fig. 1. There were no significant differences among the final body weights in the MX, NP, GEN and SI treatment groups. SDM and EB treatments significantly reduced the body weight gain (Table III). Relative organ weights are given in Table III. Thyroid (P<0.001) and pituitary (P<0.05) weights in the SDM treatment group were significantly greater than those of the basal diet group. Pituitary (P<0.001), liver (P<0.05) and kidney weights (P<0.05) in the EB treatment group were significantly greater than those of the basal diet group. Thyroid weights were also increased in the EB group albeit without statistical significance. MX, NP, GEN and SI-supplemented groups did not exhibit changes in any organ weights, including the thyroid.

Histopathology (Table IV) Histopathologically, proliferative lesions of the thyroid can be classified into follicular cell hypertrophy, hyperplasia, adenoma, and carcinoma.
The SDM treatment significantly increased the occurrence of all four, as well as pituitary hyperplasias. On the other hand, MX, NP, GEN, SI or EB did not exert any significant influence. Histopathological changes were seen in the pars distalis of the pituitary following OVX. The affected cells were large with abundant, pale and eosinophilic cytoplasm and round vesicular nuclei. Uterus atrophy was also observed in the OVX rats. In addition, cytoplasmic vacuolation in the liver, hyaline droplet nephropathy in the kidney, squamous metaplasia of uterine epithelium, telangiec-tasia of pancreatic islets, keratinization of the vaginal epithelium and adenomas of the pituitary/pars distalis were observed in the EB treatment group. There were no organ weight changes or histopathological lesions in the major organs, including the thyroid, in the SI, GEN, NP or MX treatment group.

**DISCUSSION**

In the present study, SI, GEN, NP or MX did not influence thyroid carcinogenesis in a two-stage tumor model using OVX rats initiated with DHPN. These results are consistent with our previous finding for male F344 rats,\(^17\) in which there were no organ weight changes or histopathological lesions in the major organs including the thyroid after GEN, SI, NP or MX treatment under the same experimental conditions as in the present study. Since 1000 ppm of SDM was earlier found to induce thyroid proliferative lesions within 12 weeks,\(^10\) it was used as a positive control. The obvious increase in lesion development validated the experimental model applied.

Synthetic and natural estrogens have been shown to induce tumors in various organs such as the kidney,\(^18\) liver,\(^19\) pituitary,\(^20\) and mammary gland.\(^21\) In addition, estrogen is known to enhance extra-thyroidal conversion of thyroxine (T\(_4\)) to triiodothyronine (T\(_3\)) and to affect the pituitary-thyroid axis indirectly.\(^22, 23\) 17β-Estradiol can remarkably increase secretion of TSH in a dose-dependent fashion\(^24, 25\) and ovarian hyperstimulation in women is associated with elevated estradiol and T\(_3\), although decreased free T\(_4\), TSH and T\(_3\)-binding globulin.\(^26\) Fur-
Thermore, administration of estradiol to rats was found to enhance the development of thyroid proliferative lesions under the influence of a low iodine diet. Thyroid proliferative lesions were not promoted by EB in the present study, although thyroid weight was somewhat increased, suggesting a marginal effect. ER has been identified as a principal cellular target for estrogenic compounds and there is also some evidence for ER-regulated gene transcription in vitro and in vivo. It has been reported that normal and tumor thyroid cells have

Table III. Final Body and Relative Organ Weights

| Group                     | Body weight (g) | Pituitary (mg) | Thyroid (mg) | Liver (g) | Kidneys (g) | Uterus (g) |
|---------------------------|-----------------|---------------|-------------|-----------|-------------|------------|
| DHPN + SDM (1000 ppm)    | 174.0±7.9*      | 7.25±0.9*     | 89.5±28.7** | 2.77±0.11* | 0.9±0.04    | 0.023±0.005 |
| DHPN + MX (30 ppm)       | 199.3±10.9      | 5.57±0.6      | 7.1±0.9     | 2.67±0.11 | 1.1±0.07    | 0.023±0.005 |
| DHPN + Genistein (250 ppm)| 200.8±7.5      | 5.53±0.2      | 6.3±0.9     | 2.62±0.07 | 1.0±0.03    | 0.024±0.005 |
| DHPN + Genistein (25 ppm) | 200.1±4.9      | 5.50±0.6      | 7.6±1.1     | 2.57±0.28 | 1.1±0.09    | 0.028±0.005 |
| DHPN + Nonylphenol (250 ppm) | 202.0±9.0   | 5.60±0.4      | 7.5±0.7     | 2.35±0.07 | 1.0±0.02    | 0.024±0.004 |
| DHPN + Nonylphenol (25 ppm) | 196.8±9.4   | 5.19±0.4      | 7.7±0.8     | 2.36±0.08 | 1.0±0.06    | 0.022±0.005 |
| DHPN + Isoflavone (400 ppm) | 205.2±5.8   | 5.36±0.4      | 7.6±0.6     | 2.42±0.11 | 1.1±0.04    | 0.022±0.006 |
| DHPN + β-Estradiol 3-benzoate (0.5 mg) | 166.7±18.3** | 179.56±95.9*** | 10.1±1.2   | 4.00±0.32* | 1.4±0.09** | 0.290±0.070*** |
| DHPN                       | 199.5±6.3      | 5.47±0.4      | 8.1±1.1     | 2.37±0.10 | 1.0±0.05    | 0.026±0.007 |
| Control                    | 203.1±13.8     | 5.38±0.4      | 7.0±0.9     | 2.26±0.11 | 1.1±0.08    | 0.025±0.006 |

* Significantly different from the control value at \( P < 0.05 \).
** Significantly different from the control value at \( P < 0.001 \).
# Significantly different from the DHPN treatment group value at \( P < 0.05 \).
## Significantly different from the DHPN treatment group value at \( P < 0.001 \).
ERs, and estradiol might promote thyroid tumorigenesis through receptor binding. However, studies on ER expression have failed to provide clear insight into its potential role in thyroid tumor progression. Direct mitogenic action of estrogenic hormones, and catechol estrogens such as 4-hydroxyestradiol, may also play a role. In the present study, 0.5 mg of EB in cholesterol pellets induced pituitary adenomas and increased uterine weight. Thus, the dose of EB was estrogenic in the uterus and in the hypothalamic/pituitary axis, but was not sufficient to exert a major effect on the thyroid.

Pituitary adenomas, kidney tubule lesions, telangiectasia of pancreatic islets, uterus squamous metaplasia and keratinization of the vagina were also observed in the EB treatment group in this study. The kidney hyaline droplet nephropathy and liver cytoplasmic vacuolation lesions might be in line with the carcinogenicity of estrogen in these organs. The lesions observed in the pituitary, uterus and vagina are well known to be due to estrogenic effects. Telangiectasia of the pancreatic islets was also observed previously in hamsters treated with ethynyl estradiol or levonorgestrel (Furukawa, unpublished data) and seems to be related to the influence of estradiol and 2-hydroxyoestradiol, a metabolite of estrogen, on insulin secretion.

The soybean has been implicated in diet-induced goiter and several investigators have reported induction of goiter and carcinoma in rats maintained on a soybean diet with an iodine deficiency. GEN (4,5,7-trihydroxyisoflavone), a major isoflavone, inhibits thyroid peroxidase (TPO), a key enzyme in the biosynthesis of thyroid hormone. Nevertheless, in the present study, SI and GEN did not induce thyroid proliferative lesions after DHPN pre-treatment.

EECs have long been recognized to have uterotrophic activity in a variety of animal species. Isoflavones are among the most important phytoestrogens, which may have estrogenic and antiestrogenic effects. In the present study, SI did not induce uterine adenomas, in line with the earlier observation that 148 mg/day isoflavone-rich soy protein isolate did not induce proliferation in endometrial and mammary tissues in postmenopausal female macaques (Macaca fascicularis). On the other hand, dietary GEN (375 and 750 μg/g) increased uterine wet and dry weights (P<0.05) in OVX, but not in immature rats. While 210 ppm GEN produced a large uterotropic effect in immature OVX rats, this was not observed in the study by Casanova et al. using pregnant Sprague-Dawley females fed 200 or 160 ppm GEN. In the present case, the administration of 250 ppm GEN did not affect uterine weight. However, the experimental protocols and dosages differed among reports, and this could provide an explanation for the apparent anomalies. The nonlinear dose-response of the uterotrophic effect of dietary GEN clearly warrants further study.

NP, a weak estrogenic compound, has been shown to have dose-dependent uterotrophic effects in immature female rats when given i.p. at levels of 20 and 50 mg/kg or p.o. at a level of 50 mg/kg, although 2000 ppm of NP did not induce significant uterine changes in a subchronic study using rats and in a multigeneration reproductive study. In the present study, the dose of 250 ppm NP administered in the diet corresponds to approximately 11–44 mg/kg/day in rats. It did not induce uterine or thyroid lesions. In addition to the fact that the dosing regimen of NP, exposure route, or experimental method in our studies was different from those in previous studies, species as well as strain variability in response to compounds with estrogenic activity is well documented. Furthermore, OVX females are less susceptible to estrogenic chemicals than immature or prepupal females. Indeed, 50 mg/kg of NP induced a significant increase in uterine weight in prepupal rats, but was ineffective for stimulating a similar response in OVX adult rats in a 3-day uterotrophic assay. Based upon the dose-response data for NP, the dose of 250 ppm does not appear to pose any problem regarding uterotrophic or thyroid carcinogenic effects.

MX formed during chlorination of drinking water is a very potent bacterial mutagen and a mammalian cell clastogen, and tumor-promoting effects have been reported. In a recent carcinogenicity study using rats showed MX to be definitely carcinogenic, in a dose-dependent manner, when given in drinking water at doses of 5.9, 18.7 and 70.0 ppm for 104 weeks. The most prominent tumors were follicular adenomas and carcinomas of the thyroid glands in both sexes, the mechanism apparently being a direct carcinogenic effect rather than promotion through increasing the TSH level. However, MX did not induce thyroid proliferative lesions in this study.

In conclusion, the EECs, SI, GEN and NP, and a possible goitrogenic chemical, MX, did not exert any promotional effect on thyroid carcinogenesis in OVX rats initiated with DHPN under the present experimental conditions. The results are also in good agreement with our findings in our recent study using male F344 rats.

ACKNOWLEDGMENTS

We thank Dr. Naohide Kinae (University of Shizuoka) for providing MX. This work was supported by a Grant-in-Aid (H11-Seaikatsu-018) for Research on Environmental Health from the Ministry of Health and Welfare of Japan.
REFERENCES

1) EDSTAC. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report. August (1998).
2) Capen, C. C. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol. Pathol., 25, 39–48 (1997).
3) O’Connor, J. C., Frame, S. R., Davis, L. G. and Cook, J. C. Detection of thyroid toxicants in a tier 1 screening battery and alterations in thyroid endpoints over 28 days of exposure. Toxicol. Sci., 51, 54–70 (1999).
4) Paloyan, E., Hofmann, C., Prinz, R. A., Oslapas, R., Shah, K. H., Ku, W. W., Ernst, K., Smith, M. and Lawrence, A. M. Castration induces a marked reduction in the incidence of thyroid cancers. Surgery, 92, 839–848 (1982).
5) Farbota, L., Hofmann, C., Oslapas, R. and Paloyan, E. Sex hormone modulation of serum TSH levels. Surgery, 102, 1081–1087 (1987).
6) Hofmann, C., Oslapas, R., Nayyar, R., McCall, A. and Paloyan, E. Testosterone enhancement of thyroid carcinoma in rats: the role of TSH. Surgery, 100, 1078–1087 (1986).
7) Mori, M., Naito, M., Watanabe, H., Takeichi, N., Dohi, K. and Ito, A. Effects of sex difference, gonadectomy, and estrogen on N-methyl-N-nitrosourea induced rat thyroid tumors. Cancer Res., 50, 7662–7667 (1990).
8) Padmapria, A. A., Just, G. and Lewis, N. G. Synthesis of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, a potent mutagen. Can. J. Chem., 63, 828–832 (1985).
9) Chang, Y. C., Nair, M. G., Santell, R. C. and Helferich, W. G. Microweave mediated synthesis of genistin. J. Agric. Food Chem., 42, 1869–1871 (1994).
10) Mitsumori, K., Onodera, H., Takahashi, M., Shimo, T., Yasuhiro, K., Kitaura, K., Takahashi, M. and Hayashi, Y. Effect of thyroid stimulating hormone on the development and progression of rat thyroid follicular cell tumors. Cancer Lett., 92, 193–202 (1995).
11) Ikeda, T., Nishikawa, A., Imazawa, T., Kimura, S. and Hirose, M. Dramatic synergism between excess soybean intake and iodine deficiency on the development of rat thyroid hyperplasia. Carcinogenesis, 21, 707–713 (2000).
12) Cai, Q. and Wei, H. Effect of dietary genistin on antioxid-ant enzyme activities in SENCAR mice. Natr. Cancer, 25, 1–7 (1996).
13) Odum, J., Pyrah, I. T., Soames, A. R., Foster, J. R., Van Miller, J. P., Joiner, R. L. and Ashby, J. Effects of p-nonylphenol (NP) and diethylstilbestrol (DES) on the Alderley Park (Alpk) rat: comparison of mammary gland and uterus sensitivity following oral gavage or implanted mini-pumps. J. Appl. Toxicol., 19, 367–378 (1999).
14) Nishikawa, A., Kinae, N., Furukawa, F., Mitsui, M., Enami, T., Hasegawa, T. and Takahashi, M. Enhancing effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) on cell proliferation and lipid peroxidation in the rat gastric mucosa. Cancer Lett., 85, 151–157 (1994).
15) Nishikawa, A., Furukawa, F., Lee, I., Kasahara, K., Tanakamaru, Z., Nakamura, H., Miyauchi, M., Kinae, N. and Hirose, M. Promoting effects of 3-chloro-4-(dichloro-romethyl)-5-hydroxy-2(5H)-furanone on rat glandular stom-ach carcinogenesis initiated with N-methyl-N′-nitro-N′- nitrosoguanidine. Cancer Res., 59, 2045–2049 (1999).
16) Botts, S., Jokinen, M. P., Isaac, K. R., Meuten, D. J. and Tanaka, N. Proliferative lesions of the thyroid and parathyroid glands. E-3. In “Guides for Toxicologic Pathology,” pp. 1–12 (1991). STP/ARP/AFIP, Washington, DC.
17) Son, H. Y., Nishikawa, A., Ikeda, T., Nakamura, H., Miyauchi, M., Imazawa, T., Furukawa, F. and Hirose, M. Lack of modifying effects of environmental estrogenic compounds on the development of thyroid proliferative lesions in male rats pretreated with N-bis(2-hydroxypropyl)nitrosamine (DHPN). Jpn. J. Cancer Res., 91, 899–905 (2000).
18) Liehr, J. G., Roy, D., Ari-Ulubelen, A., Bui, Q. D., Weisz, J. and Strobel, H. W. Effect of chronic estrogen treatment of Syrian hamsters on microsomal enzymes mediating formation of catechol estrogens and their redox cycling: implications for carcinogenesis. J. Steroid Biochem., 35, 555–560 (1990).
19) Machishi, H., Higashi, S., Hibusami, H., Nakashima, K., Kawarada, Y. and Mizumoto, R. Role of activation of ome- thine decarboxylase and DNA synthesis on ethynylestradiol-induced hepatocarcinogenesis. Carcinogenesis, 16, 2965–2971 (1995).
20) Stone, J. P., Holzman, S. and Shellabarger, C. J. Neoplastic responses and correlated plasma prolactin levels in diethyl-stilberol-treated ACI and Sprague-Dawley rats. Cancer Res., 39, 773–778 (1979).
21) Shull, J. D., Spady, T. J., Synder, M. C., Johansson, S. L. and Pennington, K. L. Ovary intact, but not ovariectomized female ACI rats treated with 17β-estradiol rapidly develop mammary carcinoma. Carcinogenesis, 18, 1595–1601 (1997).
22) Galton, V. A. Thyroxine metabolism and thyroid function in the pregnant rat. Endocrinology, 82, 282–290 (1968).
23) Chen, H. J. and Wallish, P. G. Effects of estradiol benzoate on thyroid-pituitary function in female rats. Endocrinology, 103, 1023–1030 (1978).
24) D’Angelo, S. A. and Fisher, J. S. Influence of estrogen on the pituitary-thyroid system of the female rat: mechanisms and loci of action. Endocrinology, 84, 117–222 (1969).
25) Miller, W. L., Knight, M. M. and Gorski, J. Estrogen action in vitro: regulation of thyroid stimulating and other pituitary hormones in cell cultures. Endocrinology, 101, 1455–1460 (1977).
26) Muller, A. F., Verhoeff, A., Mantel, M. J., De Jong, F. H. and Berghout, A. Decrease of free thyroxine levels after controlled ovarian hyperstimulation. J. Clin. Endocrinol. Metab., 85, 545–548 (2000).
27) Money, W. L. Chemical carcinogenesis and sex hormones in experimental thyroid tumors. In “Thyroid Cancer,” ed. C. E. Hedinger, pp. 140–149 (1969). Springer-Verlag, New York.
28) Fujimoto, N., Sakai, Y. and Ito, A. Increase in estrogen receptor levels in MNU-induced thyroid tumors in LE rats. Carcinogenesis, 13, 1315–1318 (1992).
29) Watson, C. S., Pappas, T. C. and Gametchu, B. The other
oestrogen receptor in the plasma membrane: implications for the actions of environmental oestrogens. *Environ. Health Perspect.*, **103**, 41–50 (1995).

30. Bolger, R., Wiese, T. E., Ervin, K., Nestich, S. and Checovich, W. Rapid screening of environmental chemicals for oestrogen receptor binding capacity. *Environ. Health Perspect.*, **106**, 551–557 (1998).

31. Cook, J. C., Kaplan, A. M., Davis, L. G. and O’Connor, J. C. Development of a tier 1 screening battery for detecting endocrine-active compounds (EACs). *Regul. Toxicol. Pharmacol.*, **26**, 60–68 (1997).

32. Yane, K., Kitahori, Y., Konishi, N., Okaichi, K., Ohnishi, T., Miyahara, H., Matsuanga, T., Lin, J. C. and Hiasa, Y. Expression of the estrogen receptor in human thyroid neoplasms. *Cancer Lett.*, **84**, 59–66 (1994).

33. Lim, S. K., Won, Y. J., Lee, H. C., Huh, K. B. and Park, Y. S. A PCR analysis of ERalpha and ERbeta mRNA abundance in rats and the effect of ovarectomy. *J. Bone Miner. Res.*, **14**, 1189–1196 (1999).

34. Jaklic, B. R., Ruslin, J. and Ghosh, B. C. Estrogen and progesterone receptors in thyroid lesions. *Ann. Surg. Oncol.*, **2**, 429–434 (1995).

35. Lee, C., Kao, H., Lin, H., Peng, F. and Chi, C. Estrogen receptors and glucocorticoid receptors in human well-differentiated thyroid cancer. *Int. J. Mol. Med.*, **2**, 229–233 (1998).

36. Nandi, S., Guzman, R. C. and Yang, J. Hormones and mammary carcinomaogenesis in mice, rats, and humans: a unifying hypothesis. *Proc. Natl. Acad. Sci. USA*, **92**, 3650–3657 (1995).

37. Newbold, R. R. and Liehr, J. G. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. *Cancer Res.*, **60**, 235–237 (2000).

38. Leminger, J. R. and Jokinen, M. P. Oviduct, uterus, and vagina. In “Pathology of the Fischer Rat. Reference and Atlas. Endocrine System,” ed. G. A. Boorman, S. L. Eutis, M. R. Elwell, C. A. Montgomery, Jr. and W. F. MacKenzie, pp. 176–184 (1983). Academic Press, Inc., San Diego, CA.

39. Faure, A., Aerts, L., Aouari, M. H., Sutter, B. C. and Van Miller, J. P. Subchronic toxicity (90-day) study with para-nonylphenol in rats. *Regul. Toxicol. Pharmacol.*, **26**, 172–178 (1997).

40. Chapin, R. E., Delaney, J., Wang, Y., Lanning, L., Davis, B., Collins, B., Mintz, N. and Wolfe, G. The effects of 4-nonylphenol in rats: a multigeneration reproduction study. *Toxicol. Sci.*, **52**, 80–91 (1999).

41. Adams, N. R. Phytostrogens. In “Toxicants of Plant Origin,” ed. P. R. Cheeke, pp. 23–51 (1989). CRC Press, Boca Raton, FL.

42. Ashby, J., Odum, J. and Foster, J. R. Activity of raloxifene in immature female rats. *Bull. Environ. Contam. Toxicol.*, **57**, 341–348 (1996).

43. Laws, S. C., Carey, S. A., Ferrell, J. M., Bodman, G. J. and Cooper, R. L. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. *Toxicol. Sci.*, **54**, 154–167 (2000).

44. Cuney, H. C., Mayes, B. A., Rosica, K. A., Trutter, J. A. and Van Miller, J. P. Subchronic toxicity (90-day) study with para-nonylphenol in rats. *Regul. Toxicol. Pharmacol.*, **26**, 172–178 (1997).

45. Meier, J. R., Blazak, W. F. and Knohl, R. B. Mutagenic and clastogenic properties of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone: a potent bacterial mutagen in drinking water. *Environ. Mol. Mutagen.*, **10**, 41–24 (1987).

46. Futhara, C., Yamashita, M., Inoue, N. and Matsushima, T. Genotoxicity and cell proliferative activity of 3-(chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in rat glandular stomach. *Water Sci. Tech.*, **25**, 342–345 (1992).

47. Komulainen, H., Kosma, V. M., Vahtinen, S. L., Virtanien, T., Kaliste-Korhonen, E., Lotjonen, S., Tuominen, R. K. and Tuomisto, J. Carcinogenicity of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone in the rat. *J. Natl. Cancer Inst.*, **89**, 848–856 (1997).