Modifying Effects of Endocrine Disrupting Chemicals on N-bis (2-hydroxypropyl) Nitrosamine and Sulfadimethoxine-Induced Thyroid Carcinogenesis in Rats

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Abstract: To clarify whether 17β-estradiol 3-benzoate (EB), methoxychlor (MXC), atrazine (ATR), or bisphenol A (BPA) have any modifying effects on relatively late stages of thyroid carcinogenesis, six-week old female castrated F344 rats in Experiments 1 and 2 first received a single subcutaneous injection of 2000 mg/kg body wt of N-bis (2-hydroxypropyl) nitrosamine (DHPN) and, starting one week later, were given drinking water containing 1000 ppm sulfadimethoxine (SDM) for 8 weeks. Then, in Experiment 1, cholesterol pellets containing 0.5 mg EB were subcutaneously embedded and renewed every 4 weeks for 25 weeks. Controls received basal diet alone. In Experiment 2, rats of the control, MXC, ATR, or BPA group received diet containing no supplement, 1000 ppm MXC, 400 ppm ATR, and 10000 ppm BPA, respectively, for 27 weeks. Thyroid follicular cell hyperplasias, adenomas, and/or carcinomas were induced in all of the groups. In Experiment 1, the incidence of adenomas in the EB group was significantly increased, as compared to the corresponding control group value. In Experiment 2, the incidences of carcinomas in the MXC and BPA groups were significantly lower than in the corresponding control group. Serum estrogen levels and the PCNA labeling index for carcinomas in the EB group were significantly elevated but there was no clear alteration in serum thyroid related hormone levels. Serum T3 levels in the MXC and ATR groups were significantly reduced, whereas serum T4 was increased in these as well as the BPA group. No significant fluctuation in serum TSH levels was observed in these treated groups. The results of the present studies suggest that EB with strong estrogenic activity, but not MXC and BPA with only weak estrogenic activity or ATR, exerts promoting effects on thyroid carcinogenesis in rats. (J Toxicol Pathol 2001; 14: 121–128)

Key words: endocrine disrupter, thyroid, carcinogenesis, 17β-estradiol, rat

Introduction

Chemicals that disrupt the endocrine system and might impair functions of endocrine organs in the living body due to exertion of hormonal effects are generically called endocrine disrupting chemicals (EDCs). Since the late 1990’s, such chemicals that have estrogenic actions like female sex hormones have received a great deal of attention1,2. Major influences are considered to be in the reproductive system. However, EDCs also have the potential to impact on all other endocrine organs that express estrogen receptors. Therefore, investigation of whether EDCs impair their functions is a high priority using whole body approaches.

It is known that increased TSH-secretion due to failure of the negative feedback mechanism greatly contributes to thyroid carcinogenesis3,4. It has also been reported that synthesized 17β-estradiol 3-benzoate (EB), a typical EDC, enhances thyroid tumorigenesis in a two-stage thyroid carcinogenesis model using N-methyl-N-nitrosourea (MNU) as an initiator in female ovariectomized rats under conditions of iodine deficiency5. As the underlying mechanism, it was suggested that direct action of EB on the estrogen receptors was responsible. However, this remains to be confirmed and the question of whether other EDCs with less potent estrogenic activity might also exert enhancing effects remains to be clarified.

In the present study, employing a modified two-stage thyroid carcinogenesis model6 using N-bis (2-hydroxypropyl) nitrosamine (DHPN) as an initiator and
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sulfadimethoxine (SDM) as an amplifier we examined whether EB, a strong estrogen, and three other EDCs have any modifying effects on thyroid carcinogenesis in female ovariectomized rats. The compounds were methoxychlor (MXC), an organochlorine insecticide having weak estrogenic action7, atrazine (ATR), a weed killer having weak anti-estrogenic activity 8, and bisphenol A (BPA), a plasticizer contained in styrene containers9.

Materials and Methods

Female F344 rats, 4 weeks old, were purchased from Charles River Japan Inc. (Kanagawa, Japan) and housed at a maximum of 5 to a polycarbonated cage with white chips as bedding in an air-conditioned animal room (room temperature, 23 ± 2°C; relative humidity, 55 ± 5%; lighting cycle, 12 hr. light/12 hr. dark cycle). All animals were handled according with the guideline for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Pulverized basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and distilled drinking water were provided ad libitum. Animals without any abnormal findings after a 1-week acclimatization period were selected for the present experiments. Two separate experiments were performed in different seasons. In both, six-week old female F344 rats that were castrated at the age of 5 weeks received a single subcutaneous injection of 2000 mg/kg body wt of DHPN (Nakarai Tesque Inc, Kyoto, Japan) and, starting one week later, were given drinking water containing 0.1% sulfadimethoxine (SDM) (Sigma, St. Louis, MO, U.S.A) for 8 weeks to amplify the proliferative activity of thyroid focal lesions induced by DHPN.

Immediately after the end of 8-week treatment of SDM, a total of 15 rats were selected for experiment 1. Five rats were allocated to group 1 (control group) and 10 rats to group 2 (EB group), subjected to subcutaneous embedding of cholesterol pellets containing 0.5 mg EB, which were renewed every 4 weeks until week 27 from the first embedding. Since no significant elevation of serum estrogen levels was observed in our preliminary study in which diet containing 0.5 ppm ethinylestradiol (EE) was given to ovariectomized F344 rats for 27 weeks, we adopted subcutaneous embedding of EB in the present study. EB pellets were made by putting EB (Wako chemical, Osaka, Japan) dissolved in olive oil together with cholesterol powder into medical silicone tubes (SH No. 2) of 2.0–3.0 mm in diameter (Kaneka Medix, Osaka, Japan). The tubes were cut into 1cm long pellets for subcutaneous embedding. Renewal every 4 weeks maintained significantly elevated serum estrogen levels, as compared to non-treated rats in a preliminary experiment (data not shown). Both groups received pellet diet (CRF-1) and distilled water ad libitum throughout the 27-week treatment period (Fig. 1). However, five of 10 rats died or were killed in extremis by week 24 because of deterioration of general condition. Therefore, the treatment of EB was completed at week 25. At the completion of the experiment (35 weeks from the castration), all surviving animals were sacrificed and measured for hormone levels in the blood and organ weights, as well as pathological examination of the thyroid.

For experiment 2, a total of 57 rats were selected after DHPN initiation and 8-weeks treatment with SDM. Twelve rats were allocated to the control group and 15 rats each to

![Fig. 1. Experimental protocol.](image-url)
the EDC-treated groups. Groups 1 (control group), 2 (M XC group), 3 (AT R group) and 4 (B PA group), received basal diet (Pulverized CRF-1) alone, diet containing 1000 ppm M XC (Sigma), 400 ppm ATR (Wako chemical, Osaka, Japan) or 10000 ppm B PA (Wako chemical, Osaka, Japan) for 27 weeks, respectively. These doses were selected based on the maximum tolerated doses in long-term toxicity studies (MTD). Throughout the treatment period, basal or pulverized diet and distilled water were given ad libitum (Fig. 1). At the completion of the experiments (37 weeks from the castration), all surviving animals were subjected to measurement of hormone levels in the blood and organ weights, as well as pathological examination of the thyroid (Fig. 1).

For both Experiments 1 and 2, at the end of the scheduled treatment period, blood samples were taken from the abdominal aorta under ethyl ether anesthesia for all animals, and serum was collected to determine serum levels of estradiol, T3, T4, and TSH by radioimmunoassay using a DPC estradiol double antibody kit (Diagnostic Products Corporation, L.A, U.S.A.), a Coat-A-Count Canine T3 kit (Diagnostic Products Corporation), and a rat thyroid stimulating hormone (rTSH) [superscript: 125] I] assay System (Amersham Pharmacia Biotech, Buckinghamshire, England), respectively. After weighing each pituitary, thyroid, liver, and uterus, thyroid, liver, and uterus, thyroids were fixed in 10% neutral buffered formalin, routinely processed for embedding in paraffin, sectioned at 4–5 µm, and stained with hematoxylin and eosin (H-E) for microscopic examination. The number and the incidence of histopathologically-diagnosed thyroid proliferative lesions was recorded for both right and left lobes on single sections. Thyroid proliferative lesions were classified using the diagnostic criteria proposed by Boorman et al., Hyperplasia was characterized by multifocal cystic growth of follicular cells with increased amounts of colloid. Adenomas demonstrated expansive growth of follicular epithelial cells with basophilic cytoplasm and slightly atypical nuclei, readily distinguished from the surrounding thyroid tissues. Carcinomas were accompanied by invasion to the thyroidal capsule or extrathyroidal tissues. Immunohistochemical staining using a mouse monoclonal antibody to proliferating cellular nuclear antigen (PCNA) (DAKO Japan, Kyoto, Japan) was performed. At a dilution of 1:100 with avidin-biotin peroxidase complex kits (ABC, DAKO Japan), 3,3′-diaminobenzidine (DAB) as the chromogen and hematoxylin for counterstaining. The numbers of PCNA-positive cells were counted for 500–1000 cells in each type of thyroid proliferative lesion, namely follicular cell hyperplasias, adenomas, and carcinomas, which were randomly selected. PCNA-labeling indices were calculated as the ratios of positive cells per 1000 cells.

Statistical analysis

Data for body and organ weights, serum estradiol and thyroid-related hormone levels, the numbers of proliferative lesions of thyroids, and PCNA labeling indices were used to generate mean and standard deviation values, and intergroup differences were then analyzed with the Student’s t-test. Data for incidences of thyroid proliferative lesions were analyzed using the Fisher’s exact test.

Results

In Experiment 1, during the experimental treatment period from week 1 to 24, 5 out of 15 rats in the EB group died or were killed in a moribund condition because of deterioration of general condition due to pituitary tumors, especially prolactin secreting-lesions attributable to EB treatment. The body weight values in the EB group were significantly lower than those for the control group throughout the experimental treatment period, and the final body weights were also significantly lowered. In Experiment 2, during the experimental treatment period from week 1 to 27, none of the rats in any of the experimental groups died. However, body weights for the MXC, ATR, and B PA groups were significantly lower than those in the control group throughout the treatment period. Especially, the body weight gain in the MXC group was remarkably depressed, as compared to the other treated groups (Fig. 2, Table 1).

In Experiment 1, with regard to the pituitary, liver, and uterus, both absolute and relative weights in the EB group were significantly increased as compared to the control group. The thyroid weight of the EB group was higher than that of the control group, but this was not statistically significant because of large standard deviations. In Experiment 2, the relative pituitary weights in the MXC, ATR, and B PA groups were significantly increased as compared to the control group, the absolute and relative liver weights were significantly decreased and increased, but thyroid weights were not altered. The absolute and relative uterine weights in the MXC but not the ATR and B PA groups were significantly increased, as compared to the control group (Table 1).

Thyroid follicular cell hyperplasias, adenomas, and/or carcinomas were induced in all of the groups. In Experiment 1, the incidence and multiplicity of thyroid follicular cell adenomas in the EB group were significantly increased, as compared to the control group, although values for thyroid follicular cell hyperplasias and carcinomas were not affected. In experiment 2, the incidences of thyroid follicular cell carcinomas in the MXC and B PA groups were significantly lower than in the control group, but there were no significant differences regarding thyroid follicular cell hyperplasias and adenomas (Fig. 3, Fig. 4). Edema of the uterus was observed in the EB and MXC groups. Furthermore, lung adenomas attributable to the treatment of DHPN were occasionally observed in all of the treated groups, but there was no significant difference in the incidence between treated and control groups.

In Experiment 1, the PCNA labeling index for carcinomas in the EB group was significantly higher than that in the control group. In Experiment 2, there was no...
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significant modification in the index for any type of proliferative lesion in any of the treated groups as compared to the control group (Fig. 5).

In Experiment 1, serum estradiol levels in the EB group were significantly higher than in the control group, but no significant influence on serum thyroid related hormone levels was observed. In Experiment 2, serum T3 levels of the MXC and ATR groups were significantly decreased, but serum T4 levels were increased in these two groups and the BPA group, as compared to the control group. No significant variation in serum estradiol and TSH levels was observed in any of the treated groups.
Discussion

Clearly a great variety of the EDCs exist in our environment. Recently, MXC and ATR have been shown to have estrogenic and anti-estrogenic activities, respectively, and therefore classified as the EDCs\(^{16-19}\). BPA is also known to be a weakly estrogenic substance\(^{20,21}\), widely utilized as a material in plastic products, for example tableware made of polycarbonates. Furthermore, Krishman\( et al.\) reported that BPA was released from flasks made of this material during autoclaving, and pointed out the risk of oral exposure\(^{9}\).

Nevertheless, with regard to EDC effects on thyroid, it is generally accepted that thyroid hormone receptors play the major role in toxicity rather than their estrogenic influence. This has been stressed in discussions of screening to detect thyroid hormone like effects\(^{22}\).

The results of Experiment 1 clarified that long term subcutaneous embedding of EB pellets significantly elevates

<table>
<thead>
<tr>
<th>Group</th>
<th>Hyperplasia</th>
<th></th>
<th>Adenoma</th>
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<th>Carcinoma</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
<td>Multiplicity</td>
<td>Incidence</td>
<td>Multiplicity</td>
<td>Incidence</td>
<td>Multiplicity</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
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<td></td>
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<tr>
<td>Control</td>
<td>5/5 (100)</td>
<td>8.00 ± 2.55(^a)</td>
<td>1/5 (20)</td>
<td>0.20 ± 0.45</td>
<td>3/5 (60)</td>
<td>1.00 ± 1.00</td>
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<tr>
<td>EB</td>
<td>5/5 (100)</td>
<td>7.80 ± 3.77</td>
<td>5/5 (100)*</td>
<td>13.00 ± 1.41**</td>
<td>4/5 (80)</td>
<td>0.80 ± 0.45</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12/12 (100)</td>
<td>1.88 ± 1.60</td>
<td>0/12 (0)</td>
<td>0</td>
<td>10/12 (83)</td>
<td>1.18 ± 0.40</td>
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<td>MXC</td>
<td>15/15 (100)</td>
<td>1.61 ± 1.02</td>
<td>2/15 (13)</td>
<td>1.00 ± 0.0</td>
<td>6/15 (40)*</td>
<td>1.17 ± 0.41</td>
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<tr>
<td>ATR</td>
<td>15/15 (100)</td>
<td>1.78 ± 1.02</td>
<td>0/15 (0)</td>
<td>0</td>
<td>8/15 (53)</td>
<td>1.20 ± 0.42</td>
</tr>
<tr>
<td>BPA</td>
<td>15/15 (100)</td>
<td>1.78 ± 1.07</td>
<td>0/15 (0)</td>
<td>0</td>
<td>4/15 (27)**</td>
<td>1.00 ± 0.97</td>
</tr>
</tbody>
</table>

\(^a\): Mean ± S.D.

\(*, **\): Significantly different from the corresponding Control group at \(p<0.05\) and 0.01, respectively.
serum estrogen levels and enhances the development of thyroid focal proliferative lesions. This means that the rat two-stage thyroid carcinogenesis model used here thus proved useful for screening purposes. However, Hiasa et al. reported that the thyroid follicular adenomas were induced in male Wistar rats at 20 weeks after 2800 mg/kg DHPN initiation. On the other hands, no thyroid proliferative lesions were induced in male Fischer rats in the same experimental design. In this respect, there seems to be a strain difference in the induction of thyroid tumors by the treatment of DHPN. Therefore, such a strain difference should be taken into account for the future improvement of our experimental protocol.

Ito et al. reported that thyroid tumors were enhanced and the level of estrogen receptors (ER) in thyroid tumors were increased by subcutaneous administration of EB under conditions of iodine deficiency in rats given MNU initiation. Furthermore, Fujimoto et al. suggested that the enhancement was caused by the combined effects of stimulation of ERs in the thyroid and elevation of TSH. In the present two experiments, no significant influence on serum TSH levels was noted at the terminal killing. Therefore, the results suggest that EB exerts enhancing effects on thyroid proliferative lesions in rats via direct stimulation of estrogen receptors. With enhancement of uterine carcinogenesis of EDCs, it is known that they typically bind to receptors and the resultant estrogen receptor-ligand complexes subsequently interact with estrogen response elements (ERE), and recruit transcription factors, resulting in enhanced mRNA synthesis and gene expression for polypeptide growth factors such as epidermal growth factor (EGF) and transforming growth factor α (TGFα) that are responsible for stimulating cell proliferation. We have found that treatment with EE enhances the development of uterine tumors in heterozygous p53 deficient CBA mice initiated with ethyl nitrosourea (ENU), as compared to treatment with MXC. These data suggest that substances which have stronger estrogenic action cause stronger enhancement of tumor development via estrogen receptors. The case of thyroid proliferative lesion development with EB in our present experiment may be similar. However, since liver and pituitary weights were increased in EB treated rats of our study, the possibility of activation of the TSH secreting-cells in the pituitary or acceleration of the T4-excretion in the liver should be taken into account as enhanced mechanism thyroid proliferative lesions in rats.

In Experiment 2, relative uterine weights in the MXC and BPA groups that were castrated for removing endogenous estrogenic activities were significantly increased, suggesting estrogenic effects. However, the incidences of thyroid follicular cell carcinomas were significantly decreased, as compared to the control group. The ATR group also exhibited a tendency for decrease in the incidence of thyroid follicular cell carcinomas, though no significant difference was recognized in relative uterine weights. In addition, although serum T3 or T4 levels in all EDCs treated groups differed from the control group values, the fluctuation of these two parameters was not consistent, suggesting a lack of any toxicological significance. Moreover, serum TSH levels in the treated

<table>
<thead>
<tr>
<th>Group</th>
<th>Hyperplasia (%)</th>
<th>Carcinoma (%)</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.03 ± 0.13*</td>
<td>4.80 ± 1.35</td>
</tr>
<tr>
<td>EB</td>
<td>1.13 ± 0.35</td>
<td>10.50 ± 3.63*</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>0.46 ± 1.67</td>
<td>11.66 ± 4.90</td>
</tr>
<tr>
<td>MXC</td>
<td>0.19 ± 0.33</td>
<td>8.18 ± 7.73</td>
</tr>
<tr>
<td>ATR</td>
<td>0.03 ± 0.12</td>
<td>8.37 ± 3.77</td>
</tr>
<tr>
<td>BPA</td>
<td>0.04 ± 0.20</td>
<td>6.55 ± 2.69</td>
</tr>
</tbody>
</table>

a): Mean ± S.D.
*: Significantly different from the Control group in Experiment 1 at \( p<0.05 \).

### Table 4. Changes of Serum Levels of Estradiol, T3, T4 and TSH in Rats Treated with EB or EDCs after DHPN initiation and SDM Amplification

<table>
<thead>
<tr>
<th>Group</th>
<th>Estradiol (pg/mL)</th>
<th>T3 (ng/dL)</th>
<th>T4 (µg/dL)</th>
<th>TSH (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.02 ± 1.89*</td>
<td>117.48 ± 5.48</td>
<td>4.16 ± 1.80</td>
<td>7.31 ± 0.69</td>
</tr>
<tr>
<td>EB</td>
<td>305.30 ± 88.15**</td>
<td>160.20 ± 87.69</td>
<td>3.89 ± 2.63</td>
<td>6.83 ± 0.53</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.50 ± 0</td>
<td>102.95 ± 13.77</td>
<td>4.05 ± 0.79</td>
<td>8.55 ± 4.71</td>
</tr>
<tr>
<td>MXC</td>
<td>2.50 ± 0</td>
<td>83.21 ± 17.02**</td>
<td>5.15 ± 0.45**</td>
<td>6.44 ± 0.39</td>
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<tr>
<td>ATR</td>
<td>2.76 ± 0.58</td>
<td>86.00 ± 9.03**</td>
<td>4.87 ± 0.33**</td>
<td>7.31 ± 2.12</td>
</tr>
<tr>
<td>BPA</td>
<td>2.75 ± 0.54</td>
<td>112.23 ± 100.55**</td>
<td>5.93 ± 0.33**</td>
<td>7.35 ± 0.68</td>
</tr>
</tbody>
</table>

a): Mean ± S.D.
**: Significantly different from the corresponding Control group at \( p<0.01 \).
groups demonstrated no significant variation and PCNA labeling indices of thyroid follicular cell hyperplasias and carcinomas did not alter. Thus oral exposure of EDCs in this experiment did not appear to stimulate cell proliferation in thyroid proliferative lesions. The lack of enhancement of development of thyroid proliferative lesions, or effects on thyroid related hormones, suggested endocrine disrupting effects were not sufficiently strong, although we did obtain evidence in line with the previously reports that MXC and BPA are estrogenic, and ATR has anti-estrogenic activities.

In conclusion, the results suggest that only EB with strong estrogenic activity exerts enhancing effects on thyroid proliferative lesions in rats. In contrast, MXC and BPA, with weak estrogen activity and the anti-estrogen, ATR, proliferative lesions in rats. In contrast, MXC and BPA, strong estrogenic activity exerts enhancing effects on thyroid anti-estrogenic activities.

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References


27. Sumpter JP, Jobling S, and Tylor CR. Oestrogenic chemicals