EFFECTS OF DIETHOFENCARB ON THYROID FUNCTION AND HEPATIC UDP-GLUCURONYLTRANSFERASE ACTIVITY IN RATS

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Abstract......To examine the mechanism and toxicological significance of thyroidal tumor observed slightly in a long-term rat study with diethofencarb (isopropyl 3,4-diethoxycarbonilate), male Sprague-Dawley rats were fed diethofencarb in diets at concentrations of 0, 5,000 or 20,000 ppm for 3 months. Examinations mainly for thyroid functions including thyroid uptake of $^{125}$I, serum thyroid hormone and thyroid stimulating hormone (TSH) level, hepatic UDP-glucuronyltransferase (UDP-GT) activity and histopathological examination in thyroid were performed at week 13.

Decreases of body weights and food consumptions were observed at and above 5,000 ppm. Under these conditions, decrease of serum free T$_4$ and increase of serum TSH level were observed only at 20,000 ppm, concurrently with liver weight increase at and above 5,000 ppm and increase of hepatic UDP-GT activity at 20,000 ppm. However, no compound related effects were noted in thyroid weight, thyroid uptake of $^{125}$I and gross or histopathological examination in thyroid.

These results indicate that the administration of diethofencarb leads to an increase in UDP-GT activity and acceleration of thyroid hormone excretion from the liver. The acceleration causes a decrease in serum free T$_4$ level, triggering the feedback mechanism of the pituitary gland, promotion of TSH release and consequently an increase in serum TSH level. Thus, the slightly higher incidence of thyroid follicular cell tumors observed in the chronic and oncogenicity study with non-genotoxic diethofencarb is considered to be caused by these weak pituitary-thyroid hormonal imbalances. The toxicological significance in humans is extremely low according to the well established facts that the chronic TSH stimulant would not induce thyroid

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tumors in humans and humans may be less sensitive than rats in regard to the response to goitrogenic stimuli.

Key words: Diethofencarb, thyroid hormone, TSH, UDP-glucuronyltransferase, thyroid tumor, mechanism.

INTRODUCTION

Diethofencarb is a novel and selective fungicide developed by Sumitomo Chemical Company, Ltd. which is specifically effective against the benzimidazole-resistant strains of various fungi, including Botrytis spp. and Venturia spp. Combined chronic toxicity and oncogenicity study in Sprague-Dawley rats with diethofencarb (Cox and Brusick, 1989) revealed a slight increase in the incidence of thyroid tumor at the highest dietary maximum tolerated dose of 5,000 ppm, although a battery of mutagenicity studies (Salmonella typhimurium or Escherichia coli reverse mutation test, Chinese hamster lung cells gene mutation test, Chinese hamster ovary cells chromosomal aberration test or sister chromatid exchange test and micronucleus test in male mice) of diethofencarb showed negative results. Moreover, 18-month long-term study in ICR mice have demonstrated no oncogenicity in any organ or tissue even at the highest dietary maximum tolerated dose of 10,000 ppm (Cox, 1988). The 3-month subacute feeding study in rats showed only liver effects (increased liver weights and centrilobular hypertrophy) at and above 3,000 ppm with no histopathological findings on thyroid up to the highest dose of 10,000 ppm (Hosokawa, et al., 1986).

Similar rat liver effects and thyroid changes have been reported for the microsomal enzyme inducers (Oppenheimer et al., 1968; Bastomsky, 1974, 1977; Comer et al., 1985; Sanders et al., 1988; McClain et al., 1989). The mechanism of this thyroid change by these microsomal enzyme inducers has been shown to be a secondary or compensatory one in response to an increased rate of hepatic metabolism and clearance of thyroid hormones. Thyroid hormones are known to be metabolized especially in liver and kidney by various reactions and about half of the hormones is excreted into bile in rats (Hill et al., 1989). Glucuronidation represents the primary reaction in excretion of thyroid hormones in rats (Akoso et al., 1982). UDP-glucuronyltransferase (UDP-GT) is an enzyme involved in the reaction. The decreases in blood thyroid hormone levels trigger the feedback mechanism in the pituitary gland, promoting thyroid stimulating hormone (TSH) release from the pituitary gland and leading to an increase in blood TSH level (Emerson et al., 1989). The finding that administration of TSH caused rapid proliferative response of rat thyroid gland (Bybee and Tuffery, 1989) suggests prolonged elevations of TSH induce an increase of follicular cell tumors.

The objective of this investigation was to characterize the mechanisms and its toxicological significance of diethofencarb-induced histopathological changes in
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thyroid especially from the hormonal aspects. This paper presents the results of thyroid uptake of $^{125}$I, serum thyroid hormone and TSH level, hepatic UDP-GT activity and histopathological examination in thyroid when male Sprague-Dawley rats were fed diethofencarb in diet at 0, 5,000 and 20,000 ppm for 3 months.

MATERIALS AND METHODS

Chemicals: Diethofencarb, isopropyl 3,4-diethoxycarbanilate, was synthesized by Sumitomo Chemical Co., Ltd. (Osaka, Japan) and was 98.2 % pure by HPLC. Na$^{125}$I and $[^{125}]$-Thyroxine (46.6 $\mu$Ci/nmol) were purchased from Amasham (Buckinghamshire, UK). Thyroxine, UDP-glucuronic-acid and $\beta$-glucuronidase were purchased from Sigma Chemical Co. (St. Louis, USA).

Animals and housing: Male Sprague-Dawley derived CD rats purchased from Charles River Inc. (Kanagawa, Japan), approximately 4 weeks old upon arrival, were acclimatized for 1 week to laboratory conditions with temperature at 24±2°C, relative humidity at 55±10 %, lighting cycles of 12 hr (8:00–20:00) and frequency of ventilation 10 times or more/hr prior to the start of the study. The animals were approximately 5 weeks old at the start of the treatment. Feed and water were available ad libitum using stainless feeder and through nozzles of automated water supply system, respectively. Rats were housed in aluminum cages by 3 during the period of quarantine and by 2 during study period in a room equipped with barrier system.

Diet preparation: Diethofencarb was administered in mixture with basal diet sterilized by irradiation with $^{60}$Co (Oriental Yeast Co., Tokyo, Japan). Since the test compound was stable within the basal diet for 4 weeks (Hosokawa et al., 1986), the mixture was prepared at intervals of 2 weeks. The premix was made by blending with appropriate amounts of the test compound and basal diet in a mortar and pestle. This premix and additional quantity of diet were then blended in a mixer (SAN-EI Seisakusho Co., Tokyo, Japan). The mixtures were tested for the homogeneity of the test compound at the beginning of administration, and the concentrations of the test compound were checked every month (Sumika Chemical Analysis Service Ltd., Osaka, Japan). The homogeneity of the compound in the diet was acceptable (coefficient of variation, within 5 %). The contents of diethofencarb in the diet were found to be within ±10 % of the target concentrations, that is, the results of analysis were satisfactory.

Experimental design: Male rats (20 animals/dosage group) were orally administered diethofencarb in diet at the concentrations of 0, 5,000 or 20,000 ppm for 13 weeks. The dosage of 5,000 ppm at which an small increase in the incidence of thyroid tumor was observed in combined chronic toxicity and oncogenicity study in rats was employed as a low dose. In addition, the dose of 20,000 ppm which was considered to be sufficient as high dose to be employed in a subacute toxicity study was used as the highest dose level.
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Clinical symptoms, body weights, food consumptions: Clinical symptoms and body weights of all rats were monitored once a week. On the day of autopsy, body weights of all sacrificed rats were recorded. Food consumptions for 7 continuous days were examined once a week per cage.

Thyroid iodine uptake: Ten rats of each group were injected intraperitonealy with Na\textsuperscript{125}I in physiological saline solution (about 800 nCi/0.2 ml). At 24 hr after the injection, rats were killed by exsanguination and the thyroids were removed. After weighing the organ weight, radioactivity of \textsuperscript{125}I in the thyroid was measured by the gamma-counter (Packard Instrument Co., U. S. A.).

Serum TSH, T\textsubscript{3} and T\textsubscript{4} concentrations: Ten rats of each group were sacrified by depapititation and the blood was collected from the neck of animals to remove the serum by centrifugation. Serum TSH level was determined by NIADDK rat TSH radioimmunoassay kit (reference preparation; rTSH-PR-2) supplied by Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, U.S.A.. Serum total T\textsubscript{3}, free T\textsubscript{3}, total T\textsubscript{4} and free T\textsubscript{4} levels were measured in Special Reference Laboratory, Tokyo, Japan using Solid Phase RIA Kit (Dainabot Co., Tokyo, Japan, Ciba-Corning Diagnostic Co., Medfield, U. S. A. or Amasham Corp., U. K.).

Autopsy, organ weights and histology: After blood collection, the liver and thyroids were isolated and examined macroscopically. The wet liver weights were measured while the thyroid weights were measured after fixation in 10% neutral buffered formalin. Hematoxylin and eosin-stained paraffine embedded sections of thyroids were examined under light microscope.

Hepatic UDP-GT activity: The livers from 10 animals of each group were removed, weighed and homogenized individually in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.154 M KCl with Potter-Elvehjem homogenizer. The homogeneate was centrifuged at 10,000×G for 20 min at 4°C and resulting supernatant fraction was centrifuged at 105,000×G for 60 min at 4°C. UDP-GT activity toward T\textsubscript{4} as substrate was determined for the resulting microsomal fractions by the method of Comer et al. (1985). Protein content was measured using the Protein Assay Kit (Biorad, U. S. A.) against bovine serum albumin as a standard.

Statistical analysis: Analysis of variance in one-way classification was performed for body weight, food consumption, uptake of \textsuperscript{125}I, serum thyroid hormone level, hepatic UDP-GT activity and organ weight. Any data showing significant differences at a level of 5% were further tested for any significant difference from the control group using the LSD method. Serum TSH level was analyzed using above test method and Mann-Whitney's U test for determination of any significant difference from the control group.

RESULTS

Clinical Symptoms and Body Weights:
Localized hair loss at the trunk of the body as the test compound related change
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![Graph showing the effects of diethofencarb treatment on male rats' body weights over 90 days.](image)

**Fig. 1.** Effects of diethofencarb treatment in male rats on body weights for 13 weeks. Rats were fed diethofencarb in diet at 0, 5,000 or 20,000 ppm.

was observed in the 20,000 ppm group from Day 1 to 23. Figure 1 shows body weight changes. Lower levels of body weights were observed from Day 5 to the end of administration for the 20,000 ppm group and Day 12 to the end of administration for the 5,000 ppm group. Body weight gains during the administration period at 5,000 and 20,000 ppm corresponded to 92 and 84% of the control, respectively.

*Food Consumptions and Compound Intake:*

Decreased food consumptions were observed at Weeks 1, 5, 9 and 10 for the 20,000 ppm group and at Weeks 3, 4, and 10 for the 5,000 ppm groups (data not shown). The average amount of test compound intake for high and low dose groups was 1,280 and 308 mg/kg body weight/day, respectively.

*Thyroid Weights and Thyroid Iodine Uptake:*

Table 1 shows the effects on thyroid weight, thyroid-to-body weight ratios and thyroid iodine uptake. Increased relative thyroid weights were observed at 20,000 ppm with no significant change in absolute thyroid weight. No test compound related effects were noted in the thyroid uptake of $^{125}$I.

*Serum T₃, T₄ and TSH Concentrations:*

Serum free T₄ and TSH concentrations were significantly increased at 20,000 ppm treated with diethofencarb compared to controls. No test compound related changes were observed in T₃, T₄ and free T₃ (Table 2).
Table 1. Effects of diethofencarb treatment in male rats on thyroid weights. thyroid-to-body weight ratios and thyroid iodine uptake for 13 weeks.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Final body weight (g)</th>
<th>Thyroid weight (mg)</th>
<th>Thyroid / body weight (mg%)</th>
<th>^131I uptake /thyroid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>500±25</td>
<td>22±3.0</td>
<td>4.3±0.57</td>
<td>8.3±2.1</td>
</tr>
<tr>
<td>5,000</td>
<td>464±42*</td>
<td>21±3.3</td>
<td>4.5±0.68</td>
<td>8.2±1.5</td>
</tr>
<tr>
<td>20,000</td>
<td>440±34**</td>
<td>22±2.3</td>
<td>5.0±0.60*</td>
<td>8.0±2.8</td>
</tr>
</tbody>
</table>

a : Rats were fed diethofencarb in diet at 0, 5,000 or 20,000 ppm.
Ten animals/group were sacrificed by decapitation. Values represent mean ± SD.
b : Concentration of diethofencarb in diet.
c : ^131I uptake expressed as % of administered dose.
* : Significantly different from 0 ppm (P<0.05)
** : Significantly different from 0 ppm (P<0.01)

Table 2. Effect of diethofencarb treatment in male rats on serum T4, freeT3, T3, freeT3 and TSH level for 13 weeks.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>T4 (μg/dl)</th>
<th>freeT4 (ng/dl)</th>
<th>T3 (ng/dl)</th>
<th>freeT3 (pg/ml)</th>
<th>TSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.7±0.6</td>
<td>2.6±0.32</td>
<td>0.7±0.1</td>
<td>1.2±0.3</td>
<td>2.7±1.7</td>
</tr>
<tr>
<td>5,000</td>
<td>4.4±0.8</td>
<td>2.6±0.39</td>
<td>0.8±0.1</td>
<td>1.3±0.3</td>
<td>5.4±6.1</td>
</tr>
<tr>
<td>20,000</td>
<td>4.0±0.7</td>
<td>2.2±0.33**</td>
<td>0.8±0.1</td>
<td>1.2±0.1</td>
<td>5.9±6.5*</td>
</tr>
</tbody>
</table>

a : Rats were fed diethofencarb in diet at 0, 5,000 or 20,000 ppm.
Ten animals/group were sacrificed by decapitation. Values represent mean ± SD.
b : Concentration of diethofencarb in diet.
* : Significantly different from 0 ppm (P<0.05, Mann–Whitney’s U-test)
** : Significantly different from 0 ppm (P<0.01)

Liver Weights and Hepatic UDP-GT Activities:

Figure 2 shows the effects on liver weight and hepatic UDP-GT activity (per g liver weight basis). An increase in the absolute liver weight was observed at 20,000 ppm and an increase in the relative liver weight at and above 5,000 ppm. Measurement of UDP-GT activity using T4 as a substrate demonstrated the significant increases (P<0.01) in all of the activity per body weight, per absolute liver weight and per protein content of the liver in the animals fed diethofencarb at 20,000 ppm.

Gross Observations of Liver and Thyroid Histology:

No test compound related changes were observed grossly in the liver and thyroid in autopsy nor histopathologically in the thyroid including thyroid follicular cell hyperplasia or hypertrophy (Photo. 1).
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Fig. 2. Effects of diethofencarb treatment in male rats on liver weights and hepatic UDP-glucuronyltransferase (UDP-GT) activity (per absolute liver weight basis) for 13 weeks. Rats were fed diethofencarb in diet at 0, 5,000 or 20,000 ppm. Ten animals/group were sacrificed by decapitation. Data represent mean ± SD.

**$: significantly different from 0 ppm (P<0.01).

DISCUSSION

Diethofencarb orally administered to male rats in diets at 20,000 ppm for 13 weeks increased serum TSH and decreased serum free T₄ with an increase of hepatic UDP-GT activity. This decreased T₄ from the blood triggers the feedback mechanism at the pituitary gland, promotes TSH release from the pituitary gland and results in an increase in serum TSH level. TSH is well known to promote the biosynthesis and secretion of thyroid hormones in the thyroid. Long-term TSH stimulation induces resorption of colloid from the follicular lumen, increases in epithelial cell volume, hypertrophy and hyperplasia of the follicular epithelial cells and tumors in rats fed chronically a low iodine diets (Axelrad and Leblond, 1955; Denef et al., 1981) or treated with goitrogens such as ethylenethiourea (Graham and Hansen, 1972; Graham et al., 1973; Thomas et al., 1982). These treatments decrease the serum thyroid hormone levels and increase serum TSH level (Rognoni et al., 1982; Cooper et al., 1983, 1984). Thyroid hormone treatment or hypophysectomy exerts an antagonistic effect against the above changes including thyroid tumors (Nadler et al., 1970; Jemec, 1980). These facts suggest that diethofencarb induced thyroid follicular tumor is also considered to be produced through a promotion mechanism of TSH with a view that this compound is not
mutagenic nor clastogenic in a variety of mutagenicity studies, namely, the mechanism of the tumorigenesis is considered to be non-genotoxic.

In this study, we measured thyroid uptake of $^{125}\text{I}$ as a functional test of the thyroid and to see whether the direct antithyroid effect is observed as with ethylene:hiourea, namely an effect primarily inhibiting the organification of iodide in the thyroid, inducing inhibition of the thyroid hormone synthesis and causing hypothyroidism, resulting in the thyroid uptake of iodide (Graham and Hansen, 1972). No significant difference was observed between the diethofencarb-treated groups and the control group. This suggests that the thyroid function is maintained in an almost normal condition, and that a decrease in serum free $T_4$ level produced by the test compound is induced by a mechanism different from the direct effect mentioned above.
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Thyroic hormones are known to be converted to various metabolites at peripheral tissues of the whole body especially in liver and kidney such as deiodination, decarboxylation, deamination and various conjugational reactions, and it is well known that about half of the hormones is excreted into bile in rats (Hill et al., 1989). Glucuronidation represents the primary reaction in excretion of thyroid hormones in rats (Akoso et al., 1982). UDP-GT is an enzyme involved in the reaction. Our investigations for the diethofencarb induction of UDP-GT activity in the liver using T₄ as substrate revealed a significant increase only at 20,000 ppm in diet after 13 weeks in agreement with the absolute liver weight increase at the same dose level in this study or the hypertrophy of smooth endoplasmic reticulum of hepatocyte observed under electron microscope at 10,000 ppm in other 3-month subacute feeding study (Hosokawa et al., 1986). This enzyme has been shown to be induced by various hepatic microsomal enzyme inducers including phenobarbital and polycyclic aromatic hydrocarbons such as benzpyrene, 3-methylcholanthrene, polychlorinated biphenyls and TCDD (Goldstein and Taurog, 1968; Bastomsky and Papapetrou, 1973; Bastomsky and Murthy, 1976; Bastomsky, 1977; Collins and Capen, 1977; McClain et al., 1989). McClain et al. (1989) found that 4-week treatment with 100 mg/kg phenobarbital increased liver weight (1.4 fold), T₄-UDP-GT activity per wet weight (2.2 fold) in rats. Bastomsky and Murthy (1976) observed that 11-day treatment with polychlorinated biphenyls (250 ppm in diet) markedly increased the activity per wet liver weight (5 fold) in rats. In comparison with these hepatic enzyme inducers, the extent of UDP-GT induction by diethofencarb is small in accord with a slight increase in the incidence of thyroid tumor observed only at the highest dietary maximum tolerated dose of 5,000 ppm in the chronic and oncogenicity study with diethofencarb (Cox and Brusick, 1989).

In humans, the thyroxine-binding globulin, the major carrier protein, has an exceedingly higher affinity for T₄. This specific carrier protein is absent and thyroxine-binding prealbumin and albumin transport thyroid hormone in rodents. Therefore, more thyroid hormone is free of protein binding and is subject to metabolism and removal from the body in rats (Hill et al., 1989). The hepatic enzyme inducers probably enhance further the metabolism of thyroxine in rats. In the human study conducted by Ohnhaus et al. (1981), phenobarbital (100 mg daily for 14 days) did not affect the serum T₄, T₃, or TSH levels. A decrease in serum T₄ levels was observed after treatment with either a combination of phenobarbital plus rifampicin or a combination of phenobarbital plus antipyrine, however, these treatments had no effect on serum T₃ or TSH levels (Ohnhaus and Studer, 1983). They concluded that a decreased serum T₄ level is probably affected by increased bililial excretion in human treated with hepatic enzyme inducers and induction sufficient to increase antipyrene clearance by at least 60 % was required before a change in steady state hormone levels occurred. These data indicate that thyroid hormone imbalances via hepatic enzyme induction in rats are probably more sensitive than in humans. Epidemiologic studies of patients treated with therapeutic doses of
phenobarbital have reported no increase in risk for the development of thyroid tumor (Clemmensen and Hjallgrim-Jensen, 1977; Olsen et al., 1989).

It may be concluded from these facts that administration of diethofencarb leads to an increase in UDP-GT activity and acceleration of thyroid hormone excretion from the liver. The acceleration causes a decrease in serum free $T_4$ level, triggering the feedback mechanism of the pituitary gland, promotion of TSH release from the pituitary gland and consequently an increase in serum TSH level. However, diethofencarb was considered to have only a minimal effect on the thyroid through hormones because no compound related effects were observed in the thyroid weight, the thyroid uptake of $^{123}$I and histopathological examination of the thyroid, although a slight increase in UDP-GT activity in the liver and a decrease in serum free $T_4$ level and an increase in serum TSH level were observed only at 20,000 ppm in which a marked decrease of body weight was observed. These findings suggest that the relatively higher incidence of thyroid follicular cell tumors observed in the rat chronic and oncogenicity study with non-genotoxic diethofencarb is caused by these weak pituitary-thyroid hormonal imbalances (TSH stimulation) induced by the activation of thyroxine metabolizing enzyme. The toxicological significance in humans is extremely slight and it is unlikely that diethofencarb would increase thyroid tumor in humans.

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