Changes in thyroid function during development of thyroid hyperplasia induced by kojic acid in F344 rats

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To clarify the mechanism of tumorigenesis by kojic acid (KA), dose and time dependence of iodine uptake in the thyroid gland and serum thyroid stimulating hormone (TSH) and thyroid hormone levels were investigated in F344 rats fed a diet containing 2% KA. After 4 weeks, thyroid hyperplasia was apparent in males, associated with a decrease in 125I uptake into the thyroid gland to only 3% of that in controls. The serum triiodothyronine (T3) and thyroxine (T4) levels dropped to 0.36 ng/ml and 1.7 µg/dl from the initial values of 0.61 ng/ml and 4.0 µg/dl and TSH increased seven times to 15 ng/ml. In females, the effects on thyroid weight and 125I uptake were less prominent, although the changes in serum T3, T4 and TSH levels were similar to those in males. Time-dependent changes in serum T3, T4 and TSH levels correlated with the inhibition of iodine uptake in the thyroid. Inhibition of organic iodine formation was only observed after 3 weeks treatment. On return to the control diet, normal serum T3, T4 and TSH levels became evident within 48 h in both sexes. These data suggest that KA interrupts thyroid function, primarily by inhibiting iodine intake, consequently causing a decrease in serum T3 and T4. Increased TSH from the pituitary gland in turn stimulates thyroid hyperplasia, which is reversible on withdrawal of KA.

Introduction

Kojic acid (KA) [5-hydroxy-2-(hydroxymethyl)-4-pyrone] is widely used as a food additive to prevent enzymatic browning, as well as in cosmetics. In addition, KA is present in traditional Japanese foods such as miso, soy sauce and sake, albeit at very low concentrations, since it is produced by various strains of Aspergillus, Penicillium and Acetobacter (1–3).

Recently, KA was found to induce sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells in the presence or absence of a rat liver S9 mix (4–6), in spite of the fact that it had earlier been proven not to be mutagenic in eukaryotic systems. A 20 month examination with continuous administration of a high dose of KA in B6C3F1 mice resulted in induction of thyroid adenomas in both sexes (7). In a further study in rats, KA clearly suppressed serum triiodothyronine (T3)/thyroxine (T4) levels (8). To better clarify the manner by which KA interferes with thyroid function, in the present study we investigated its dose- and time-dependent effects on iodine uptake and organic iodine formation (organification) in the thyroid gland and serum thyroid stimulating hormone (TSH) and thyroid hormone levels in F344 rats. In addition, persistence of changes after withdrawal was examined.

Materials and methods

Animals and KA-containing diet

KA was obtained from Nagase Biochemical Co. (Tokyo, Japan) and mixed into CRF-1 basal diet (Oriental Yeast Co., Tokyo, Japan) at various concentrations. Basal or KA-containing diet and tap water were given ad libitum. F344 rats were purchased from Charles River Co. Japan (Atsugi, Japan) at the age of 5 weeks, experiments being started at the age of 6 weeks. The animal facility was air-conditioned at 24 ± 2°C with a relative humidity of 55 ± 5%. All animal experiments were carried out following the guidelines set out by Hiroshima University in the Guide for the Care and Use of Laboratory Animals.

Experiment 1. Groups of nine male animals received 0 (control), 0.008, 0.03, 0.125, 0.5 or 2.0% KA-containing diet for 4 weeks. Twenty-four hours before the end of the experiment, four animals in each group received 0.2 ml/100 g body wt Na125I (s.p. act. 579 Mbsq/ml; Amersham Pharmacia Biotech, Uppsala, Sweden) at a concentration of 2.5 × 105 c.p.m./ml (0.1 M) in saline. Animals were killed under ether anesthesia and the thyroids were dissected, weighed and investigated for 125I uptake. The remaining five animals in each group were killed on the same day. Blood was collected from the abdominal artery and serum was stored at −20°C for hormone assay. The thyroid glands were removed, fixed in 10% buffered formalin and processed for wax embedding and sectioning. Sections were stained with hematoxylin and eosin (HE) for histopathological assessment.

Experiment 2. Male and female rats were divided into eight and four groups, respectively, each consisting of eight animals, and given control or 2.0% KA-containing diet. Groups were killed at weeks 1, 2, 3 and 4 for males and at weeks 2 and 4 for females. Half of the animals served for investigation of 125I uptake and the other half for hormonal and histological examinations as in experiment 1.

Experiment 3. Male rats were divided into six groups, each consisting of eight animals, and given control and 2.0% KA-containing diet for 4 weeks. At the end of this treatment period, KA diet was replaced with control basal diet for 0, 6, 12, 24 and 48 h. Groups were then killed and examined as in experiments 1 and 2, except that 125I was injected 12 h before death.

Measurement of 125I uptake and the organic 125I formation ratio in the thyroid gland

The radioactivity of whole thyroid glands was measured with a γ counter (Wizard 1480; Amersham Pharmacia Biotech). To determine organic 125I formation, each thyroid was homogenized in 0.5 ml of 0.1 M NaCl, 1 mM KI, mixed with an equal volume of 10% trichloroacetic acid and centrifuged at 800 g for 10 min. The pellets were resuspended in 5% trichloroacetic acid and centrifuged again. Radioactivity of the pellets over the total 125I uptake was calculated as the organic iodine formation ratio.

Serum T3, T4 and TSH

Amarex-MT3 and Amarex-MT4 assay kits (Oso Clinical Diagnostic Co., Tokyo, Japan) were employed to determine serum T3 and T4 concentrations. TSH was measured with NIDDK reagents following the recommended protocol. The antigen was iodinated by the lactoperoxidase method. The second antibody, anti-rabbit IgG, was kindly provided by the Institute for Molecular and Cellular Regulation, Gunma University.
Table I. Thyroid weights, iodine uptake, organic iodine formation ratios and serum T₃, T₄ and TSH for rats treated with various doses of KA

<table>
<thead>
<tr>
<th>KA dose (%)</th>
<th>Thyroid weight (mg)</th>
<th>¹²⁵I uptake (10⁶ c.p.m./mg thyroid)</th>
<th>Organic ¹²⁵I formation ratio (%)</th>
<th>T₃ (ng/ml)</th>
<th>T₄ (µg/dl)</th>
<th>TSH (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.8 ± 0.8</td>
<td>2.43 ± 0.27</td>
<td>73.5 ± 2.6</td>
<td>0.61 ± 0.04</td>
<td>3.70 ± 0.12</td>
<td>13.6 ± 0.62</td>
</tr>
<tr>
<td>0.008</td>
<td>10.8 ± 0.9</td>
<td>1.85 ± 0.09</td>
<td>79.5 ± 2.8</td>
<td>0.57 ± 0.05</td>
<td>3.62 ± 0.10</td>
<td>11.7 ± 1.03</td>
</tr>
<tr>
<td>0.03</td>
<td>9.5 ± 0.6</td>
<td>1.63 ± 0.08a</td>
<td>76.0 ± 0.7</td>
<td>0.62 ± 0.03</td>
<td>3.53 ± 0.12</td>
<td>11.8 ± 0.97</td>
</tr>
<tr>
<td>0.125</td>
<td>13.3 ± 0.8a</td>
<td>0.90 ± 0.06a</td>
<td>73.2 ± 1.3</td>
<td>0.58 ± 0.03</td>
<td>3.33 ± 0.05</td>
<td>14.3 ± 0.39</td>
</tr>
<tr>
<td>0.5</td>
<td>23.9 ± 1.5b</td>
<td>0.34 ± 0.07b</td>
<td>74.2 ± 2.4</td>
<td>0.72 ± 0.03</td>
<td>3.35 ± 0.14</td>
<td>17.6 ± 2.03</td>
</tr>
<tr>
<td>2.0</td>
<td>90.2 ± 8.2b</td>
<td>0.04 ± 0.001b</td>
<td>39.7 ± 5.4b</td>
<td>0.36 ± 0.06b</td>
<td>1.71 ± 0.18b</td>
<td>58.2 ± 9.32b</td>
</tr>
</tbody>
</table>

Mean ± SEM (n = 4, for hormone data n = 5).

a,b Significantly different from the control value at *P < 0.05 and **P < 0.01.

**Statistical analysis**

Student's t-test was applied for the statistical analysis.

**Results**

**Effects of 4 weeks treatment with various doses of KA on thyroid weights, ¹²⁵I uptake, organic ¹²⁵I formation and serum hormone levels**

The diet containing >0.125% KA increased thyroid weight in a dose-dependent manner (Table I). The weight in the 2.0% group reached nine times the control value. ¹²⁵I uptake into the thyroid was more sensitive to KA treatment, being significantly suppressed at 0.03%. Organic ¹²⁵I formation was, however, interrupted only in the highest dose group. Serum T₃, T₄ and TSH levels were also only affected in the 2.0% KA group.

**Time-dependent changes in thyroid weights, ¹²⁵I uptake, organic ¹²⁵I formation and serum hormone levels.**

Thyroid weight increased linearly from 11 to 98 mg during 4 weeks treatment with 2% KA diet in males, while the increase was significant but less prominent in females, from 7.5 to 40 mg (Figure 1). Suppression of ¹²⁵I uptake in the thyroid glands was also time dependent. In males, it started to decrease after 1 week feeding of KA and reached only ~2% of the control at week 3, when organic ¹²⁵I formation was significantly decreased, by 50%. In females, however, the effects were far less significant; only 20% suppression of ¹²⁵I uptake was noted at week 4 (Figure 2).

Both serum T₃ and T₄ decreased to minimum levels after 2 weeks of KA treatment and recovered thereafter, although remaining lower than the control level in both sexes. Serum TSH levels started to increase at week 1 and reached a maximum at weeks 2–3 (Figure 3).

**Changes in thyroid weights, ¹²⁵I uptake, organic ¹²⁵I formation and serum hormone levels after return from KA diet to normal diet**

¹²⁵I uptake and organic ¹²⁵I formation quickly recovered when standard diet was given after 4 weeks feeding of 2% KA diet. Organic ¹²⁵I formation returned to normal after only 6 h (Figure 4). ¹²⁵I uptake per unit thyroid weight (as c.p.m./mg thyroid) rose to 70% of the control level within 24 h. Serum T₃, T₄ and TSH changed dramatically (Figure 5). Serum T₃ and T₄ were 47 and 34% of the control levels after 4 weeks feeding of KA diet. They increased to exceed the control levels between 6 and 12 h after return to standard diet, then they decreased, returning to normal levels within 48 h. The high level of serum TSH caused by the KA diet decreased to normal levels within 24 h.

**Histological findings**

The hyperplasia caused by KA treatment was of diffuse type, characterized by follicles of irregular shape lined by tall epithelial cells (Figure 6A). Two weeks after return to a normal diet, normal thyroid follicular structure was apparent in enlarged thyroid glands (Figure 6B).

**Discussion**

A previous 20 month examination in both sexes of B6C3F1 mice in our laboratory showed that continuous administration of a high dose of KA induced thyroid adenomas (7). Further investigation showed that rats are also susceptible to the carcinogenic effects of KA, with lowering of T₃/T₄ levels and interference with thyroid function, we studied the dose- and time-dependent effects of KA on iodine uptake and organic iodine formation in the thyroid gland (8,13). To further explore the manner of KA interference with thyroid function, we studied the dose- and time-dependent effects of KA on iodine uptake and organic iodine formation in the thyroid gland and serum TSH and thyroid hormone levels in F344 rats in the present study. The data clearly showed that KA suppresses iodine uptake and consequently thyroid hormone levels drop and TSH from the pituitary gland increases in turn to cause thyroid hyperplasia.

Interestingly, while interference with ¹²⁵I uptake was noted in both sexes, albeit more so in males, the suppression of organic ¹²⁵I formation was not apparent before 3 weeks of KA treatment in males and no significant decrease was found in
Kojic acid-induced thyroid hyperplasia

Fig. 2. Time-dependent changes in $^{125}$I uptake and organic $^{125}$I formation in rats receiving 2% KA diet ($n = 6$). Bars indicate SEM; *,**significant differences from relevant control values at $*P < 0.05$ and $**P < 0.01$, respectively.

Fig. 3. Time-dependent changes in serum $T_3$, $T_4$ and TSH levels in rats receiving 2% KA diet ($n = 6$). Bars indicate SEM; *,**significant differences from relevant control values at $*P < 0.05$ and $**P < 0.01$, respectively.

effect may be similar for several anions, such as thiocyanate and perchlorate, which suppress thyroid function by inhibiting the iodide pump transporting iodide from the extracellular fluid into thyroid follicular cells (14). These agents could also inhibit organic iodination at doses higher than those inhibiting iodide transport and their administration, in fact, causes thyroid hyperplasia in rats. However, our results are in contrast to findings with synthetic anti-thyroid drugs, including propylthiouracil and methimazole, which block the incorporation of iodide into tyrosine residues of thyroglobulins, probably by inactivating thyroid peroxidase (15,16).

The thyroid gland in males is known to be more susceptible to the anti-thyroid drugs discussed above than that of females in both rats and mice (15). Increase in thyroid weight caused
Fig. 5. Recovery in serum T3, T4 and TSH levels after withdrawal of the KA diet (n = 4). Bars indicate SEM; ** significant differences from relevant control values at * \( P < 0.05 \) and ** \( P < 0.01 \), respectively.

by KA was also more prominent in males than in females in the present experiment, in line with an earlier experiment with B6C3F1 mice (7). Since there were no differences in basal serum TSH levels between sexes, with a similar increase (six times the control value) due to KA treatment, the sex difference in thyroid weight increase must be explained by factors other than TSH levels. Jolin et al. also found that neither plasma TSH levels nor iodine content of the thyroid gland differed between sexes given propylthiouracil and a low iodine diet whereas goiters were more pronounced in males (17). Even in intact adult rats, conflicting results with regard to serum TSH, T3 and T4 levels and sex differences have been reported (18–20), although both androgens and estrogens are known to influence serum TSH levels (21,22). Growth hormone may have a synergistic effect on goitrogenesis by propylthiouracil (23,24) and other factors such as EGF and IGF are also able to modulate thyroid function (25,26). These could have accounted for the sex difference in the present study.

After withdrawal of KA treatment in males, thyroid function recovered very rapidly. Organic 125I formation ratio returned to almost normal levels after 6 h, along with 125I uptake in the thyroid. It is worth remarking that total uptake of 125I in the thyroid gland in the KA withdrawal group was greater than that in the controls, since the weight of the thyroid gland in the KA group was elevated nine times. Reflecting the recovery of thyroid function, both serum T3 and T4 levels increased after the withdrawal, exceeding the control level after 6–12 h and then returning to the control levels. The rapid recovery suggests that: (i) orally administered KA is quickly metabolized; (ii) the effects of KA on thyroid function are highly reversible. The increased thyroid weight caused by KA, however, persisted for at least 2 weeks after return to the normal diet, although a normal thyroid follicular structure reappeared. This result is in agreement with reports that a hyperplastic thyroid gland does not necessarily resume its normal status after withdrawal of the goitrogenic stimulus (27,28).

Our 20 month examination with continuous administration of KA in mice resulted in an increase in development of thyroid adenomas (7). KA may possess genotoxic potential in vitro and it has been reported to cause sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells (4–6). However, no induction of dominant lethal effects was observed in an experiment with rats initiated with N-bis(2-hydroxypropyl)nitrosamine (8). These results and our present findings strongly suggest that the proliferative effect of KA on the thyroid is not related to a genotoxic pathway. Although the molecular mechanisms of action need to be examined further, considering the high reversibility of its effects on the thyroid, KA or possibly derivatives might provide a new class of anti-thyroid agents.
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References


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