EFFECTS OF KOJIC ACID ON THYROIDAL FUNCTIONS IN RATS BY SINGLE-DOSE ADMINISTRATION AND IN CULTURED RAT THYROID CELLS (FRTL-5 CELLS)

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ABSTRACT — The effects of kojic acid (KA) on thyroidal function were studied by single-dose administration in rats and in cultured rat thyroid cells (FRTL-5 cells). In rats receiving a single dose of 1,000 mg/kg KA orally, the ¹²⁵I uptake from blood into the thyroid gland was significantly lower than that of the control group from 30 min to 24 hr after administration. The ¹²⁵I organification activity of the KA groups was significantly lower than control from 30 min to 6 hr after administration. However, the ¹²⁵I organification activity at 24 hr or 48 hr after administration recovered enough to be nearly comparable with the control group. In the study in FRTL-5 cells, KA inhibited iodine organification dose-dependently, but did not inhibit iodine uptake. These results suggest that the observed lower iodine uptake activity in the single-dose administration study in rats was due to the inhibition of iodine organification caused by the oral administration of KA, consequently decreasing iodine in the entire thyroid gland.

Although serum T4 showed a tendency to decrease from 2 hr to 48 hr after administration of KA, serum TSH did not show any evident change associated with KA in the single-dose administration study in rats. Based on these results, it is presumed that a massive dose or long administration period might be needed to decrease serum T4 and increase serum TSH.

From these results, it is presumed that KA affected thyroidal function when given at a massive dose or in a long administration period by inhibiting iodine organification in the thyroid.

KEY WORDS: Kojic acid, Thyroid function, Single-dose administration, Cultured rat thyroid cells (FRTL-5 cells), Iodine uptake, Iodine organification

INTRODUCTION

Kojic acid [5-hydroxy-2- (hydroxymethyl)-4-pyrone] is known to suppress the biosynthesis of melanin at melanocytes in epidermal skin by inhibiting the activity of tyrosinase, which is an enzyme involved in biosynthesis of melanin (Nakayama *et al.*, 1982; Higa, 1983). Based on this effect, the compound has been used as an active skin-whitening agent in the fields of

cosmetics and quasi-drugs (Ohyama *et al.*, 1990). In addition, KA is employed as a food additive to prevent enzymatic browning of fresh food products such as lobsters, crabs, vegetables, and meat (Uchino, 1986a, 1986b). Recently, Burdock *et al.* (2001) have reviewed the health aspects of KA in food and concluded that KA consumed in an amount and in a manner consistent with historical use did not present a safety concern (Burdock *et al.*, 2001).

As for the effect of KA on the thyroid gland, it was reported that the dietary administration of KA significantly decreased the levels of serum T3 and T4, while significantly increasing the serum TSH level and thyroid weights, and inhibiting iodine uptake and iodine organification in several studies (Hirose *et al.*, 2001; Tamura *et al.*, 1999; Fujimoto *et al.*, 1999). Recently, we have also reported thyroid weight gain and a morphological change, *i.e.*, follicular epithelial hypertrophy, observed in the thyroid of rats orally administered KA for 4 weeks (Higa *et al.*, 2000). In addition, increased ^{125}I uptake from blood into the thyroid was observed along with increased ^{125}I in the TCA-precipitation fraction in the thyroid gland. However, no apparent changes were seen in ^{125}I organification activity in the thyroid gland. As for determination of serum hormone concentration, T4 showed a decrease while TSH did not show any apparent change. These observations suggest that massive administration of KA may decrease blood T4 concentration and may enhance thyroid function compensatingly. However, the mechanism of T4 decline has yet to be clarified, since the effect of thyroid hypertrophy was too dominant. We therefore focused on a further review of the effect of KA on thyroidal functions in rats by single-dose administration and in cultured rat thyroid cells (FRTL-5 cells) in the present study.

MATERIALS AND METHODS

In vivo single-dose administration study in rats

1. Test compound, animals and housing conditions

The structural formula of KA is shown in Fig.1. KA was obtained by a fermenting method using a mutant strain of *Aspergillus sp.* owned by Sansho Seiyaku Co., Ltd. (Fukuoka, Japan).

Male F344/Du Crj rats, five-weeks-old, were purchased from Charles River Japan, Inc., (Kanagawa,

Japan), and were used for the experiment after 6-day acclimatization. The animal facility was air-conditioned at $24 \pm 2^\circ\text{C}$ with a relative humidity of $55 \pm 10\%$, and 12 light/12 dark cycle. Pellet food (MF, Oriental Yeast Co., Chiba, Japan) and tap water with sodium hypochlorite added were provided *ad libitum*.

2. Administration

Since KA influenced thyroidal function at 1,000 mg/kg dose in our previous repeat oral administration study for 4 weeks (Higa *et al.*, 2000), we selected 1,000 mg/kg as the dose level in the present study. Rats were given single-dose administration of KA suspended in 0.5% carboxymethylcellulose (Wako Pure Chemical Industries Ltd., Inc., Osaka, Japan) solution orally through a plastic gastric tube.

3. General observation

The clinical signs and mortality of the animals were checked after KA administration. Body weight was measured after administration and at autopsy. Relative thyroid weight was calculated from the measured thyroid and body weight of all animals at autopsy.

4. Determination of thyroid ^{125}I uptake and organification

A group of rats was promptly given 8×10^6 cpm/0.4 mL of $\text{Na } ^{125}\text{I}$ (450–500 MBq/ μg , NEN Life Science Products Inc., Tokyo, Japan) saline intraperitoneally after KA administration. The animals were killed at 0.5, 1, 2, 4, 6, and 24 hr after injection of $\text{Na } ^{125}\text{I}$ and blood samples collected. The thyroid gland was then removed and weighed for record. Four animals were used at each time point.

Another group of rats was given $\text{Na } ^{125}\text{I}$ at 24 hr or 48 hr after KA administration and killed 24 hr later. The animals were then treated in the same manner as the former group.

The radioactivity in the serum collected and in the thyroid we removed was measured by gamma counter (Auto Gamma Counting System MINAXI 5530; Packard Instrument Co., Meriden, USA).

A thyroid gland with 0.5 mL of cold 0.15 M NaCl -1 mM KI added was homogenized by cooling with ice. The radioactivity of the 0.1 mL homogenates was then measured. The amount of proteins contained in the homogenate fraction was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (Sigma Chemical Co., St. Louis, USA) as the standard substance. The equivalence of 10% trichloro acetic acid (TCA, Wako Pure Chemical

Fig. 1. Chemical structure of kojic acid (KA).
5-Hydroxy-2-(hydroxymethyl)-4-pyrone.

Industries Ltd., Inc., Osaka, Japan) was added to the remaining homogenate before centrifugation (1,300 ×g, 10 min). After adding 5% TCA for re-suspension, the pellet was centrifuged again to determine its radioactivity.

¹²⁵I uptake ratio and TPB (Thyroid Protein Binding) were calculated as indices of the ¹²⁵I uptake and ¹²⁵I organification activities, respectively. They were calculated by the following formula:

¹²⁵I uptake activity (% of dose);

¹²⁵I uptake ratio = (radioactivity in the thyroid / radioactivity administered to the animal) × 100

¹²⁵I organification activity (%);

¹²⁵I organification ratio = TPB (Thyroid Protein Binding) = (TCA -precipitate radioactivity / total radioactivity in the thyroid) × 100

5. Determination of serum hormone concentrations

The hormone concentrations in the serum collected at 0.5, 1, 2, 4, 6, 24, and 48 hr after a single dose of 1,000 mg/kg KA or in the reaction medium were determined. Four animals were used at each time point.

The concentrations of serum T3 and T4 were measured with the RIA tube solid phase assay (Diagnostic Products Corporation, Los Angeles, USA), and serum TSH was measured with RIA double antibody assay (Amersham, Buckinghamshire, UK).

In vitro study with FRTL-5 cells

1. Cultured cells and culture conditions

We selected cultured rat thyroid cells (FRTL-5 cells), which are used widely to analyze the mechanism of iodine transportation (Weiss *et al.*, 1984; Brown *et al.*, 1986) in the present study. FRTL-5 cells, obtained from the American Type Culture Collection, were cultured in Ham's F-12 medium (Gibco BRL Co., Rockville, USA) under 5% CO₂ in the air, at 37°C, adding the following substances: 5% calf serum (Gibco BRL Co., Rockville, USA); 10 mU/mL TSH (bovine origin, Sigma Chemical Co., St. Louis, USA); 10 µg/mL insulin (bovine origin, Sigma Chemical Co., St. Louis, USA); 10 nM hydrocortisone (Sigma Chemical Co., St. Louis, USA); 5 µg/mL apotransferrin (human origin, Sigma Chemical Co., St. Louis, USA); 10 ng/mL glycyl-L-histidyl-L-lysine acetate (Sigma Chemical Co., St. Louis, USA); and 10 ng/mL somatostatin (Sigma Chemical Co., St. Louis, USA) (Ambesi-Impiombato *et al.*, 1980; Valente *et al.*, 1983; Brown *et al.*, 1986; Chiovato *et al.*, 1994; Stuart *et al.*, 1984). As a cell-dispersed solution (CTC solution),

HBSS (-) containing 20 U/mL collagenase (Wako Pure Chemical Industries Ltd., Inc., Osaka, Japan), 0.75 mg/mL trypsin (Sigma Chemical Co., St. Louis, USA), 2 mM EGTA (Dojindo Laboratories, Kumamoto, Japan), and 2% heated chicken serum (Gibco BRL Co., Rockville, USA) was employed. Both KA and the positive control were dissolved in the reaction medium in which the concentration of the culture medium, calf serum, was lowered to 0.5%.

2. Determination of ¹²⁵I uptake activity

The reaction medium was used to determine ¹²⁵I uptake activity and ¹²⁵I organification in FRTL-5 cells. FRTL-5 cells harvested by using CTC solution were seeded onto a 24-well plate and served for study when the cells became nearly confluent. After removing the culture solution in the wells and washing with Ham's F-12 medium, 300 µl/well of 5 mM methimazole, 100 µl/well of KA solution at each concentration and 100 µl/well of the reaction medium containing 1 µM of non-radioactive NaI (Wako Pure Chemical Industries Ltd., Inc., Osaka, Japan), including approximately 2 × 10⁷ cpm/mL of Na ¹²⁵I, were added and cultured at 37°C for 2 hr. Sodium perchlorate was selected as the positive control (n=4) (Weiss *et al.*, 1984). After a two-hour incubation, the reaction was terminated by adding 50 µl/well of 1 mM NaI. The cells were washed with HBSS (+) (Gibco BRL Co., Rockville, USA) sufficiently cooled with ice and dissolved with 0.1 M NaOH to determine radioactivity by gamma counter (MINAXI 5530 Gamma Counting System; Packard Instrument Co., Meriden, USA). Cells without incubation were set as a blank.

3. Determination of ¹²⁵I organification activity

After removing the culture solution in which FRTL-5 cells became confluent, the wells were washed with Ham's F-12 medium, with 100 µl/well of KA added at each concentration or 100 µl/well of the reaction medium containing Na ¹²⁵I (about 2 × 10⁷ cpm/mL), and were cultured at 37°C for 2 hr. Methimazole was selected as a positive control (Brown *et al.*, 1986) (n = 4). After a two-hour incubation, 50 µl/well of 1 M NaOH and an equivalence of 1 mM NaI (nonradioactive) were added, followed by 1 mL of 10% trichloroacetic acid (TCA) to precipitate the proteins. After washing with 10% TCA, the radioactivity of the precipitates was determined. As the blank control, the cell in which Na ¹²⁵I, NaOH and NaI were added after two-hour incubation was used.

Both activities (%) of ¹²⁵I uptake and ¹²⁵I organification were calculated from the mean value (Ci) of

each concentration's counts (cpm) ($n = 4$), the mean cpm (Co) of the negative control in which the reaction medium was added instead of KA, and the mean cpm (CB) of the blank by the following formula:

$$^{125}\text{I uptake activity (\%)} = (\text{Ci} - \text{CB}) / (\text{Co} - \text{CB}) \times 100$$

$$^{125}\text{I organification activity (\%)} = (\text{Ci} - \text{CB}) / (\text{Co} - \text{CB}) \times 100$$

Statistical analysis

Student's *t*-test (Yoshimura, 1987) was applied for comparison with the control group at the *p* value of 0.05 or 0.01 in all cases.

RESULTS

In vivo single-dose administration study in rats

1. General observation

Although almost all of the rats showed hypoactivity 1 hr after receiving a single dose of 1,000 mg/kg KA, they recovered in about 30 min. With respect to body weight as well as absolute and relative thyroid weights, no differences were found with the controls until 72 hr after administration, when the observation time was over.

2. ^{125}I uptake activity

The ^{125}I uptake activity into the thyroid (% of dose) of the control group increased time-dependently

and remained at a constant level from 6 hr to 24 hr after administration. In contrast, the activity of the KA group was significantly lower than the control group from 30 min to 24 hr after administration (Fig. 2).

The ^{125}I uptake activity at 24 hr after injection of Na ^{125}I , following KA administration 48 hr before, recovered enough to be nearly comparable with that of the control at 24 hr after KA administration (Fig.3).

3. ^{125}I organification activity

As shown in Fig.4, the ^{125}I organification activity of the control group remained at approximately 90% from 30 min to 24 hr after administration in the control animals.

The activity of the KA group was significantly lower than control from 30 min to 6 hr after administration. However, when Na ^{125}I was given intraperitoneally at 24 or 48 hr after administration of KA, the activity recovered enough to be nearly comparable with that of the control (Fig. 5).

4. Serum T3, T4, and TSH concentrations

As shown in Table 1, serum T3 showed a tendency to increase from 30 min to 6 hr after administration of KA, while serum T4 showed a tendency to decrease from 2 to 48 hr after administration. Serum TSH level did not fluctuate in evident association with

Fig. 2. ^{125}I uptake activity in rat thyroid after single oral administration of 1000 mg/kg KA. KA and Na ^{125}I were administered simultaneously. ^{125}I uptake activity; ^{125}I uptake ratio = (radioactivity in the thyroid / radioactivity administered to the animal) $\times 100$. Each point represents the mean $\pm$ S.D. of four rats. **: $p < 0.01$ (Significantly different from control by *t*-test).

KA administration.

***In vitro* study with FRTL-5 cells**

With regard to ^{125}I uptake and organification activities, the effects on ^{125}I uptake activity by KA or sodium perchlorate in FRTL-5 cells are shown in Fig. 6. KA did not inhibit ^{125}I uptake at a concentration range from 0.1 μM to 300 μM , while the positive control, sodium perchlorate, inhibited ^{125}I uptake dose-dependently.

The effects on ^{125}I organification activity by KA or methimazole in FRTL-5 cells are shown in Fig. 7. Both KA and the positive control, methimazole, inhibited ^{125}I organification dose-dependently.

DISCUSSION

In our previous study of oral administration for 4 weeks, in which rats were given 0, 4, 15, 62.5, 250 and 1,000 mg/kg of KA, we reported that the weight increase in the thyroid gland and a morphological change, *i.e.*, hypertrophy of the thyroid follicular epithelial cells, were observed in the group given 1,000

mg/kg of KA (Higa *et al.*, 2000). In addition, ^{125}I uptake from blood into the thyroid and the ^{125}I in the TCA-precipitate fraction in the thyroid were enhanced in those rats, while ^{125}I organification activity did not change. As for the serum hormone determination, although serum T4 concentration decreased, TSH concentration did not change in the 1,000 mg/kg KA group. These changes were not observed at less than 1,000 mg/kg of KA. Based on these observations, we have speculated that thyroid hypertrophy was due to a decreased concentration of blood T4 and compensatingly enhanced thyroid function, and that the enhanced ^{125}I uptake into the thyroid and enhanced ^{125}I in the TCA-precipitate fraction in the thyroid were due to thyroid hypertrophy caused by repeatedly administered KA at a massive dose for a long period of time. However, the mechanism of decreased T4 concentration has yet to be clarified with the 4-week repeated administration method, because the influence of thyroid hypertrophy was too dominant.

We therefore focused on the time-course changes of iodine uptake into the thyroid, iodine organification in the thyroid and the concentration of serum T3, T4 and TSH

Fig. 3. ^{125}I uptake activity in rat thyroid for 24 hr after intraperitoneal injection of $\text{Na } ^{125}\text{I}$. $\text{Na } ^{125}\text{I}$ was injected intraperitoneally into rats at 0, 24 or 48 hr after single oral administration of 1000 mg/kg KA.

^{125}I uptake activity ; ^{125}I uptake ratio = (radioactivity in the thyroid / radioactivity administered to the animal) $\times 100$.

Each point represents the mean $\pm$ S.D. of four rats.

** $p < 0.01$ (Significantly different from control by *t*-test).

caused by administration of KA in the present study. In addition, the effects of KA on iodine uptake into the thyroid and iodine organification in cultured rat thyroid cells (FRTL-5 cells) were also examined.

In the single-dose administration study in rats, the ^{125}I uptake activity in the KA groups was significantly lower than that of the control group from 30 min to 24 hr after administration (Fig. 2). The ^{125}I organification activity in the thyroid of the KA group was also significantly lower than control from 30 min to 6 hr after administration. However, the figure recovered to be nearly comparable with the control at 24 hr or 48 hr after administration

(Fig. 4, 5). Based on these results, we assumed that the iodine organification ratio would recover more rapidly than the iodine uptake ratio.

The thyroid gland is comprised of thyroid epithelial cells and follicles, which are filled with colloid containing iodized thyroglobulin. The epithelial cells actively uptake iodine from the blood and organify the iodine on the colloid side of the epithelial cells or on the border. The organified iodine is then stored in the colloid (Imura, 1988; Daichi, 1995; Kloassen, 1996; Hardman and Limbird, 1996). From the results in FRTL-5 cells, namely that KA inhibited the iodine organification but did not inhibit

Table 1. Serum levels of T3, T4 and TSH in rats after single oral administration of 1000 mg/kg KA.

Time	T3 (ng/dl)		T4 ($\mu\text{g/dl}$)		TSH (ng/ml)	
	Control	KA	Control	KA	Control	KA
0.5 hr	220.5 $\pm$ 25.3	262.2 $\pm$ 6.6*	6.0 $\pm$ 0.3	6.0 $\pm$ 0.3	4.0 $\pm$ 6.0	1.7 $\pm$ 1.0
1 hr	236.5 $\pm$ 24.3	251.4 $\pm$ 42.7	6.4 $\pm$ 0.4	6.3 $\pm$ 0.9	4.9 $\pm$ 5.3	66.5 $\pm$ 77.4
2 hr	249.5 $\pm$ 27.4	277.2 $\pm$ 18.3	7.6 $\pm$ 0.3	6.2 $\pm$ 0.5**	7.5 $\pm$ 10.8	21.3 $\pm$ 40.8
4 hr	228.8 $\pm$ 20.7	312.1 $\pm$ 21.5**	6.6 $\pm$ 0.3	6.3 $\pm$ 0.5	2.2 $\pm$ 1.7	3.7 $\pm$ 1.8
6 hr	196.0 $\pm$ 16.0	254.4 $\pm$ 10.0**	6.6 $\pm$ 0.5	5.7 $\pm$ 0.7	1.0 $\pm$ 0.3	2.1 $\pm$ 0.8
24 hr	205.3 $\pm$ 33.3	201.4 $\pm$ 26.9	5.7 $\pm$ 0.5	4.7 $\pm$ 0.4*	15.3 $\pm$ 27.4	2.4 $\pm$ 1.4
48 hr	189.6 $\pm$ 20.8	198.9 $\pm$ 21.9	5.1 $\pm$ 0.5	4.7 $\pm$ 0.3	3.7 $\pm$ 1.3	2.5 $\pm$ 1.2

Each value represents the mean $\pm$ S.D. of four rats.

*: $p < 0.05$, **: $p < 0.01$ (Significantly different from control by *t*-test).

Fig. 4. ^{125}I organification activity in rat thyroid after single oral administration of 1000 mg/kg KA.

KA and Na^{125}I were administered simultaneously. ^{125}I organification activity; ^{125}I organification ratio = TPB (Thyroid Protein Binding) = (TCA precipitate radioactivity / total radioactivity in the thyroid) $\times$ 100.

Each point represents the mean $\pm$ S.D. of four rats. **: $p < 0.01$ (Significantly different from control by *t*-test).

Effects of Kojic acid on thyroidal functions in rats.

Fig. 5. ^{125}I organification activity in rat thyroid for 24 hr after intraperitoneal injection of $\text{Na } ^{125}\text{I}$.

$\text{Na } ^{125}\text{I}$ was injected intraperitoneally into rats at 0, 24 or 48 hr after single oral administration of 1000 mg/kg KA.

^{125}I organification activity ; ^{125}I organification ratio = TPB (Thyroid Protein Binding) = (TCA precipitate radioactivity / total radioactivity in the thyroid) $\times 100$.

Each point represents the mean $\pm$ S.D. of four rats.

Fig. 6. Effects on ^{125}I uptake activity by KA and Sodium perchlorate in FRTL-5 cells.

iodine uptake, it was presumed that the observed lower iodine uptake activity in the single-dose administration study in rats was due to decreased thyroglobulin in the colloid as a result of the inhibition of iodine organification caused by oral administration of KA, consequently decreasing iodine in the entire thyroid. Therefore, it is

speculated that KA seemed to inhibit iodine uptake into the thyroid.

Although serum T4 showed a tendency to decrease from 2 hr to 48 hr after administration of KA, no evident changes in serum TSH suggested that single-dose administration of KA did not decrease serum T4 enough to

Fig. 7. Effects on ^{125}I organification activity by KA and methimazole in FRTL-5 cells.

Fig. 8. Action mechanism of Kojic acid on rat thyroid function.

increase serum TSH. A massive dose or long administration period might be needed to decrease serum T4 and increase serum TSH.

Given the result that the decreased iodine uptake caused by KA was due to its inhibition of iodine organification, the action mechanism of KA on thyroidal function was considered as follows (Fig. 8): KA first inhibits iodine organification in the thyroid, and consequently the decreased iodized thyroglobulin in the colloids suppresses the formation of T3 and T4. This decreased blood T4 concentration leads to excessive TSH secretion through a negative feedback mechanism, and the thyroid

gland is then enlarged. Although KA has been known to inhibit the activity of tyrosinase and restrain the biosynthesis of melanin (Nakayama, 1982; Higa, 1983), its effect on thyroid peroxidase activity has not yet been clarified.

Hirose *et al.* (2001) have reported that lack of DNA adducts and 8-OHdG formation in the thyroid of rats administered KA is not in accordance with genotoxic potential. Humans appear to be less sensitive to perturbation of the thyroid-pituitary status than rodents. In addition, it has been reported that monkeys are less sensitive to the goitrogenic effects of peroxi-

dase inhibitors such as phenyl thiourea and sulfamethoxazole, and there have been no reports of adverse effects on the thyroid after long-term clinical use of sulfonamides, suggesting that human risk of thyroid cancer due to such agents will be very low. In addition, Burdock *et al.* (2001) have concluded that KA consumed in an amount and in a manner consistent with historical use did not present a safety concern. Since KA is absorbed, metabolized and excreted rapidly, the function of iodine organification in rats recovers as rapidly as 24 hr after KA administration. Therefore, the thyroid hypertrophy observed in rats is considered to be seen only when KA is given at a massive dose or for a long period of time.

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