Modulation of Cytochrome P450 Expression by Kojic Acid in Rats

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Abstract

Kojic acid is a naturally occurring compound used extensively as a food additive, as a food preservative, and as an ingredient in skin-lightening agents. A number of studies have reported possible toxicity and/or carcinogenicity of kojic acid, but the mechanisms involved remain unclear. We investigated effect of kojic acid on the expression of several cytochrome P450 isoforms in rats. Male F344 rats were orally administered various doses of kojic acid ranging from 0.6-1875 mg/kg bw for 14 days. High doses of kojic acid significantly decreased body weight and serum thyroxine levels, but increased liver and thyroid gland weights. The pattern of CYP2B1 protein expression in the livers of kojic acid treated rats showed kojic acid at the low dose significantly decreased the level of expression, but the medium and high doses significantly increased CYP2B1 expression. In addition, kojic acid treatment decreased CYP2E1 expression in rat livers. CYP2C11 was significantly decreased in livers of rats fed with the high dose of kojic acid. This is the first report that kojic acid influences protein expression of cytochrome P450 isozymes in rat liver, which in turn may promote liver and thyroid gland toxicity in rats.

Keywords: Cytochrome P450, Kojic acid, Liver, Rat, Thyroid gland

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การควบคุมการแสดงออกของไซโตโครมพี 450 โดยกรดโคจิคในหนูแรท

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บทคัดย่อ

กรดโคจิคเป็นสารเคมีที่พบในธรรมชาติซึ่งถูกนำมาใช้เป็นสารเติมแต่งอาหาร ถนอมอาหารและเครื่องสำอาง รายงานวิจัยเกี่ยวกับกรดโคจิคหลายฉบับที่ระบุว่ากรดโคจิคอาจทำให้เกิดความเป็นพิษและก่อมะเร็งได้ แต่ยังไม่มีรายงานที่เกี่ยวกับการเกิดกลไกเหล่านั้น งานวิจัยนี้จึงศึกษาผลของกรดโคจิคต่อการแสดงออกของเอนไซม์ในกลุ่มไซโตโครมพี 450 ในหนู ผู้วิจัยได้ทำการทดลองในหนูแรทสายพันธุ์ F344 ตัวผู้ โดยป้อนกรดโคจิคความเข้มข้นตั้งแต่ 0.6 – 1875 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว เป็นเวลา 14 วัน พบว่ากรดโคจิคที่ความเข้มข้นสูงสุดมีผลลดน้ำหนักตัวและระดับไธรอยด์ฮอร์โมนในเลือด แต่ทำให้ขนาดของตับและต่อมไธรอยด์เพิ่มขึ้นอย่างมีนัยสำคัญ นอกจากนี้กรดโคจิคที่ความเข้มข้นต่ำมีผลต่อการลดระดับการแสดงออกของไซโตโครมพี450ไอโซไซม์ชนิด2B1 แต่ที่ความเข้มข้นระดับกลางและสูงนั้นสามารถเหนี่ยวนำการแสดงออกของไอโซไซม์ดังกล่าว และพบว่าระดับของโปรตีนไซโตโครมพี450ไอโซไซม์ชนิด2E1และชนิด2C11 ลดลงในตับหนูที่ได้รับกรดโคจิคที่ความเข้มข้นสูง งานวิจัยนี้เป็นครั้งแรกที่พบว่ากรดโคจิคมีผลต่อการแสดงออกของเอนไซม์ไซโตโครมพี 450 ระดับโปรตีนในตับของหนูทดลอง ซึ่งอาจเกี่ยวข้องกับการส่งเสริมให้เกิดความเป็นพิษในตับและต่อมไธรอยด์ของหนูทดลอง

คำสำคัญ: ไซโตโครมพี 450 กรดโคจิค ตับ หนูแรท ต่อมธัยรอยด์

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Introduction

Most chemical toxicants require metabolic activation in order to induce a biological response. The production of reactive metabolites is largely dependent on primary metabolism by the cytochrome P450 enzymes and/ or secondary metabolism by the phase-2 metabolizing enzymes. The result of exposure to an environmental toxicant in terms of acute or chronic toxicity largely depends on the balance between these two processes.[1]

Cytochrome P450 (CYP)-dependent monooxygenase represents the first line of defense against toxic lipophilic chemicals as it catalyzes reactions involving the incorporation of an atom of molecular oxygen into the substrate.[2] The resulting increase in hydrophilicity facilitates further metabolic processing and excretion. Unfortunately, in this process certain chemicals are activated to their ultimate carcinogenic form rather than being detoxified. Most carcinogen activation occurs through generation of epoxides or *N*-hydroxy intermediates that are further metabolized by transferases. CYPs are most extensively expressed in the liver, although their levels of expression vary dependening on the particular P450 form.[3]

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) is a metabolic product of aerobic carbohydrate metabolism found in several microorganisms, including species of the genus *Aspergillus*. It is contained in traditional Japanese fermented foods, including miso (soybean paste), soy sauce and sake, and used as a food additive and preservative, and as a skin-whitening agent in cosmetics.[4]

Kojic acid has an indistinct toxicological profile. It has been reported to be genotoxic in several *in vitro* tests, including mutation in the Salmonella mutation assay and chromosomal aberrations test in Chinese hamster ovary cells but all of the *in vivo* genotoxicity tests were negative.[5-7] In addition, kojic acid induced thyroid adenomas and hyperplasia formation in rodents by disruption of thyroid hormones homeostasis, which is not related to the genotoxic pathway.[8-10] Several studies have reported that high concentrations of kojic acid promote hepatocarcinogenesis in rodents.[11-13] However, little is known concerning bioactivation enzymes by kojic acid. The present study aims to investigate the expression of cytochrome P450 proteins of kojic acid in rat liver.

Materials and Methods

Chemicals

Kojic acid (CAS no. 501-30-4) (purity $\geq$ 98%) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Rabbit polyclonal antibodies against rat CYP2B1, CYP2C11 and CYP2E1 were kind gifts from Prof. Yoshihiko Funae, School of Medicine, Osaka City University, Osaka, Japan.

Animals

Eight-week-old male F344 rats were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). They were housed in an animal room, maintained on a 12 h (08:00 – 20:00) light/dark cycle at a constant temperature of 23 ± 1 °C and relative humidity of 44 ± 5 % and were given free access to tap water and food. Animals were acclimated 1 week before the start of the experiment. The experiment was performed under the guidelines of the animal facility of Osaka City University Medical School.

Experimental protocol

Rats were divided into 4 groups, 4 rats per group. Group 1 was orally fed 5 ml/kg bw of 0.5% carboxymethylcellulose as a vehicle control. Groups 2 to 4 were fed various concentrations of kojic acid, 0.6, 3.0 or 1,875 mg/kg bw for 14 days. At day 15 of the experiment, all rats were anesthesized under diethyl ether. Then, livers were flushed with ice-cold perfusion buffer (1.15% KCl buffer pH 7.4 containing 1 mM EDTA and 0.25 mM phenylmethylsulfonyl fluoride) until the tissue became pale. The liver tissue was preserved in liquid nitrogen. Thyroids were removed and weighed. Blood samples were taken for analysis of liver enzyme activity and thyroid hormone level.

Cytochrome P450 isozyme level

Microsomal fractions were prepared from individual rat frozen livers following homogenization in ice-cold perfusion buffer using a Teflon-glass homogenizer and differential centrifugation. Protein amounts in the hepatic microsomes were measured by the method of Lowry using bovine serum albumin as the standard.[14] Western blotting analysis of CYP2B1, 2E1 and 2C11 were carried out as described previously.[15] Samples were diluted and mixed with sample solution, including sodium dodecyl sulfate, glycerol and mercaptoethanol, and heated to 95°C for 3 min. After polyacrylamide gel electrophoresis using prestained broad range protein as a marker, with rat CYP2B1, 2E1, and 2C11 as standards, gels were blotted to a nitrocellulose membrane and immunostained with polyclonal anti-rat CYP2B1, 2E1, and 2C11 antibodies as first antibodies and goat anti-rabbit IgG-HRP conjugate as a second antibody. Finally, the signal on the membrane

was developed via HRP coloring agent containing 4-chloro-1-napthol. The density of visible bands was measured by an NIH image J program.

Statistical analysis

All results are presented as mean ± SD. Statistical significance of differences between groups were determined by one-way analysis of variance (ANOVA) and post hoc Dunnett's multiple comparison test. *P* values < 0.05 were regarded as significant.

Results

Table 1 shows the body and organ weight results as well as food and water intake. There were no significant differences between low and medium doses of kojic acid treated groups and the vehicle control. The high concentration of kojic acid treated group presented significant weight loss and decreased food intake. The relative weights of liver and thyroid were significantly increased as compared with the control groups (Table 2). Interestingly, an increase triiodothyronine (T3) level was found in rats fed with low and medium doses of kojic acid, while a decrease in thyroxine (T4) level was found in rats treated with the high dose of kojic acid (Table 3). Thyroid stimulating hormone (TSH) in the blood could not be detected in this study. In addition, rats treated with the high dose of kojic acid had significantly decreased serum

ALP levels. The pattern of CYP2B1 protein expression in the livers of kojic acid treated rats showed that kojic acid at the low dose significantly decreased the level of expression, but the medium and high doses significantly increased CYP2B1 expression. In addition, kojic acid treatment decreased CYP2E1 expression in rat livers. CYP2C11 was significantly decreased in livers of rats fed with the high dose of kojic acid (Figure 1).

Discussion

In the present study, we demonstrated that short-term exposure to kojic acid influences hepatic cytochrome P450 protein expression in male rats. The lowest concentration (0.6 mg/kg bw) used in this study is the approximate daily intake of kojic acid in food, while the highest concentration (1875 mg/kg bw) is close to the LD50 of oral kojic acid administration in rats. The 14 day treatment with the high dose of kojic acid presented both general toxicity to male F344 rats, as observed by weight loss, and organ-specific toxicity, as observed by increased liver and thyroid gland size. Tamura T. and his colleagues showed that 4 weeks' administration of 2% kojic acid in the diet, approximately 1383.7 mg/kg bw, tended to reduce weight gain in rats.[10] We obtained similar results in that the high concentration of kojic acid decreased serum T4. These results

suggest that kojic acid may act as a goitogenic substance in rodents. It inhibits iodide uptake and iodine organification in the thyroid gland leading to disruption of thyroid hormone levels.[4,8,16] Once a negative signal from the thyroid is reduced, the pituitary gland increases production of thyroid stimulating hormone, which in turn causes thyroid enlargement. In this study we also observed low serum ALP levels caused by hypothyroidism in rats treated with the high dose of kojic acid.[17]

In addition, rats fed the high dose of kojic acid increased synthesis of CYP2B1, but displayed reduced levels of CYP2E1 and CYPC11 expression. This phenomenon was similar to that observed with phenobarbital, which can induce hepatic CYP2B1 expression in rats, causing the production of reactive oxygen species and genomic DNA oxidation.[18] It has been found that phenobarbital promoted liver tumors in rodents via induction of CYP2B1.[19] In addition, the induction of xenobiotic metabolizing enzymes in the liver is altered not only induced by exogenous substances that induce them, but also by various endogenous hormones.[20] The constitutive androstane receptor (CAR) mediates the induction of CYP2B genes by phenobarbital and other chemicals.[21] CAR is required for phenobarbital-mediated disruption of thyroid hormone homeostasis and the induction of thyroid follicular cell proliferation. CAR activation also decreased serum T4 levels in mice and increased TSH concentration, resulting in the stimulation of thyroid-follicular cell proliferation.[22] In our experiment the high dose of kojic acid induced CYP2B1 and decreased serum T4 levels, suggesting that kojic acid, at high concentrations, might activate CYP2B1 and contribute to hepatocarcinogenesis via CAR activation. In this study, we also found that hepatic CYP2E1 and CYP2C11 were significantly reduced in rats treated with the high dose of kojic acid. Some studies have found that thyroid hormones up-regulated CYP2E1 and 2C11 expression.[23] It has been reported that the suppression of CYP2C11 expression is correlated with the reduction of T4 level by several chemicals, including retinol.[24]

In conclusion, kojic acid at a high dosage might affect on cytochrome P450 protein expression by disruption of thyroid hormone homeostasis, leading to hepatotoxicity in rats.

Table 1. Body weight and intake of food and water of kojic acid treated rats

Treatment	No. of rats	Initial body weight (g)	Finial body weight (g)	Body weight change (%)	Food consumption (g/day)	Water consumption (ml/day)
Control	4	188.4± 13.4	238.1 ± 14.0	26.5 ± 1.8	14.8 + 0.49	22.53 ± 0.68
KA 0.6 mg/kg bw	4	180.6 ± 12.6	227.1 ± 7.6	25.9 ± 4.5	13.1 ± 1.75	22.60 ± 2.21
KA 3 mg/kg bw	4	185.1 ± 6.1	234.1 ± 10.0	26.4 ± 1.4	13.65 ± 0.87	21.60 ± 1.71
KA 1,875 mg/kg bw	4	187.3 ± 4.9	185.1 ± 1.8*	-1.1 ± 2.4*	10.50 ± 1.80*	19.50 ± 1.43

*Significantly different from control with $p < 0.05$

Table 2. Liver and thyroid weights of kojic acid treated rats

| Treatment	No. of rats	Liver weight		Thyroid weight	
		Absolute (g)	Relative (g%)	Absolute (mg)	Relative (mg%)
Control	4	11.96 ± 0.65	5.04 ± 0.43	22.68 ± 3.86	9.49 ± 1.22
KA 0.6 mg/kg bw	4	12.10 ± 0.82	5.34 ± 0.46	27.88 ± 4.76	12.30 ± 2.27
KA 3 mg/kg bw	4	12.30 ± 1.19	5.26 ± 0.45	26.50 ± 6.04	11.28 ± 2.19
KA 1,875 mg/kg bw	4	11.50 ± 0.84	6.12 ± 0.40*	89.43 ± 18.14*	47.78 ± 10.56*

*Significantly different from control with $p < 0.05$

Table 3. Blood biochemistry of liver function enzymes and thyroid hormone levels of kojic acid-treated rats
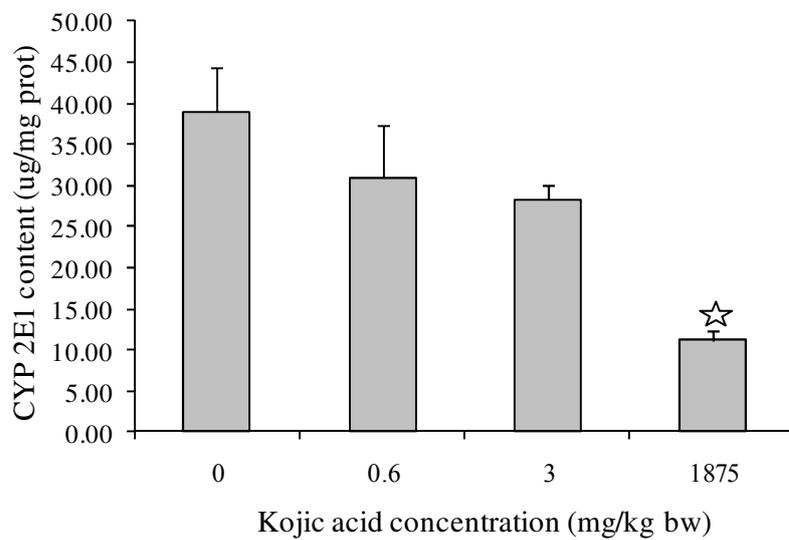
Treatment	AST (IU/l)	ALT (IU/l)	ALP (IU/l)	T3 (ng/dl)	T4 (µg/dl)
Control	109.67 ± 19.22	46.67 ± 5.69	1004.33 ± 64.66	46.67 ± 3.06	3.53 ± 0.15
KA 0.6 mg/kg bw	84.33 ± 4.16	46.00 ± 6.24	1036.33 ± 47.60	71.67 ± 2.52*	3.87 ± 0.15
KA 3 mg/kg bw	77.33 ± 7.77	45.67 ± 1.53	1039 ± 66.96	60.67 ± 3.06*	3.73 ± 0.21
KA 1,875 mg/kg bw	72.80 ± 20.68	49.40 ± 11.19	642.60 ±103.61*	43.80 ± 7.69	1.34 ± 0.59*

*Significantly difference from control with $p < 0.05$
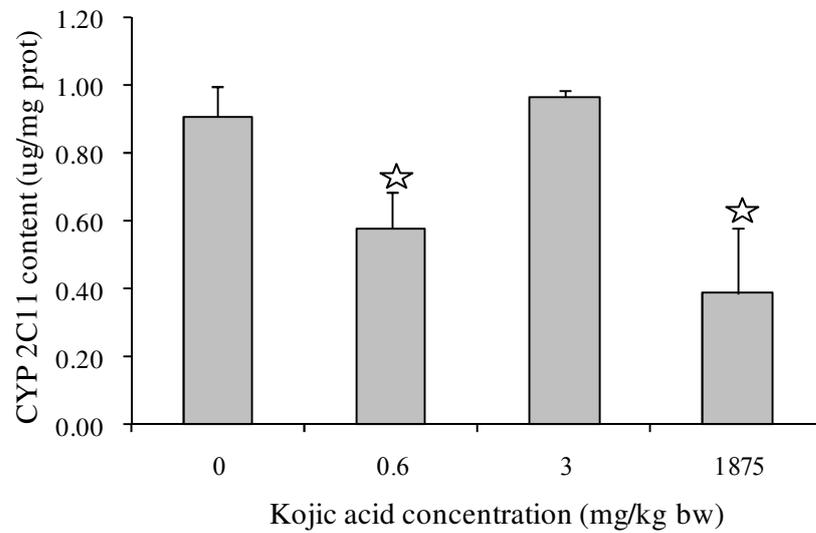
(a)
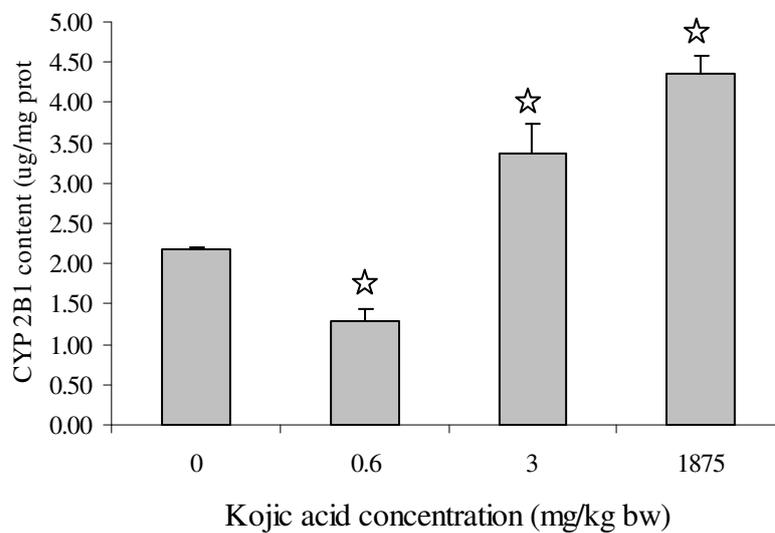
(b)

(c)

Figure 1. Cytochrome P450 levels in the liver of male rats treated with various concentrations of kojic acid, a: CYP2B1; b: CYP2E1 and c: CYP2C11.   Significantly difference from control with p < 0.05

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