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Dietary indole-3-carbinol promotes endometrial adenocarcinoma development in rats initiated with *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine, with induction of cytochrome P450s in the liver and consequent modulation of estrogen metabolism

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Indole-3-carbinol (I3C), found in cruciferous vegetables, has been shown to suppress or promote carcinogenesis depending on various animal models. Regarding its preventive effects, I3C acts as an anti-estrogen and can induce apoptosis, but precise mechanisms remain to be determined. Since I3C induces cytochrome P450 enzymes in the liver, it affects hydroxylation of estrogens and might therefore be expected to influence endometrial adenocarcinoma development. The present study was performed to clarify the effects of I3C using a rat two-stage endometrial carcinogenesis model, focusing on induction of cytochrome P450s and other estrogen-metabolic enzymes in the liver. First, to determine the estrogenic or anti-estrogenic activity, an uterotropic assay was conducted using ovariectomized Donryu rats (experiment 1). Second, to elucidate the effects on endometrial carcinogenicity, female Donryu rats initiated with a single dose of *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine into a uterine horn were fed 0 or 500 p.p.m. I3C in diets for 12 months (experiment 2). In experiment 3, similarly initiated animals received 0 or 2000 p.p.m. I3C in their diet, or 1 μg/kg 17β-estradiol (E2) or 5 μg/kg 4-hydroxyestradiol (4HE) subcutaneously twice a week for 12 months. In the uterotrophic assay, neither 500 nor 2000 p.p.m. of I3C showed any estrogenic or anti-estrogenic activity. In the two uterine carcinogenicity studies, I3C and 4HE increased incidences of uterine adenocarcinomas and/or multiplicities of uterine proliferative lesions, E2-treatment being associated with a tendency for promotion. In the liver, I3C treatment consistently elevated estradiol 2- and 4-hydroxylase activities, in particular the latter, but without effects on estradiol 16α-hydoxylase activity. mRNAs for CYP 1A1, 1A2 and 1B1 were increased by I3C treatment, with translation confirmed immunohistochemically. These results suggest that induction of the CYP 1 family in the liver and sequential modulation of estrogen metabolism to increase 4HE might play a crucial role in promoting the effects of dietary I3C on endometrial adenocarcinoma development.

Introduction

Indole derivatives are contained in cruciferous vegetables such as cabbage, broccoli, brussels, sprout and cauliflower (1). Indole-3-carbinol (I3C) is known to be an anti-estrogenic (2–4) or apoptosis-inducing compound (5), and has shown anticarcinogenic activity in a number of animal studies such as DMBA-induced rat mammary tumorigenesis (6) and spontaneous rat uterine adenocarcinoma development (7). I3C also has chemopreventive activity against benzo[*a*]pyrene-induced mouse forestomach carcinogenicity (8). It is neither cytotoxic, nor mutagenic *in vitro* (9,10), and thus I3C is a promising candidate for a chemopreventive agent against various tumors, especially estrogen-related examples. However, the compound has been documented to promote development of colon proliferative lesions in an animal model (11), and in a multi-organ rat model both inhibition and promotion were apparent, depending on the organ (12).

As for a hypothesized mechanism of chemopreventive effects of I3C, an anti-estrogenic and/or apoptosis-inducing effect have been widely accepted. In addition, the compound induces hepatic cytochrome P450s (CYPs) such as 1A1 and/or 1A2 (13–15), and increased activity of some phase I drug-metabolizing enzymes, including the CYP 1 family, can protect in some instances by increasing the rate of oxidation to less toxic metabolites (16–19). Recently I3C treatment was reported to also induce CYP 1B1 in the liver and/or other organs (20,21). In most animal species, it is well established that estradiol is metabolized by microsomal P450s in the liver and other organs/tissues, and that these enzymes therefore have the ability to modulate its effects (22–24).

In rats, CYP 1A2, 2B1/2B2 and 3A catalyze 2- or 4-hydroxylation of estradiol, mainly in the liver (24,25). In addition, evidence has recently been presented that CYP 1B1 is a major enzyme catalyzing 17β-estradiol (E2) to 4-hydroxyestradiol (4HE) (26). In the rat liver, E2 is metabolized by estradiol 2- and 4-hydroxylases into two types of catechol estrogens, 2-hydroxyestradiol (2HE) and 4HE, respectively. 2-Hydroxylation of estradiol is the dominant pathway for catechol estrogen formation (22,24), and 2HE can bind to the classical estrogen receptors, but with a markedly reduced binding affinity. This metabolite possesses much weaker hormonal potential than the parent hormone (27,28), and is not a carcinogenic agent (7,24,29). In contrast, 4HE, produced only in small amounts in the liver compared with 2HE, is hormonally active and can stimulate uterine growth by strong binding to estrogen receptors when injected into animals (24,26, 29–31). In addition, this catechol estrogen causes tumor development in the kidney in hamsters (23), and also has been implicated in uterine and mammary tumor development in human beings (32,33).

Much attention has been paid to modulation of estrogen metabolism by chemicals such as phenobarbital, dexamethasone, 3-methylcholanthrene and environmental pollutants via

Abbreviations: CYP, cytochrome; E2, 17β-estradiol; ENNG, *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine; 2HE, 2 hydroxyestradiol; 4HE, 4-hydroxyestradiol; I3C, indole-3-carbinol; PE, persistent estrus.

induction of cytochrome P450 enzymes, especially of the CYP 1 family, in the liver or other organs (26,34,35). Thus, it is hypothesized that chemicals exerting no estrogenic activity themselves but inducing CYP 1 might also modify estrogen-dependent tumor development. However, solid evidence in animal models is limited, although Kojima *et al*. (7) reported previously that dietary I3C inhibited spontaneous uterine adenocarcinoma development by increasing estradiol 2-hydroxylation activity.

Cancers of the uterine corpus, most of them being histologically endometrial adenocarcinomas, have recently been increasing in many countries of the economically developed world. The tumor development is strongly related to estrogen statement in women. In rats, spontaneous endometrial adenocarcinomas are generally very rare but Maekawa and his co-workers have described high incidences of such lesions with morphological and biological similarities to human tumors in aged Donryu rats, and shown that this is due to an age-related ovarian hormonal imbalance resulting in an increase of the serum estrogen/progesterone ratio (36–38). In addition, they have established a two-stage uterine carcinogenesis model using this rat strain to detect promotive or preventive effects of test-chemicals (39–42). The present study was conducted to clarify effects of I3C on uterine carcinogenesis using this rat model, focusing on modulation of estrogen-metabolic enzymes in the liver. In addition, estrogenic or anti-estrogenic activity of I3C on ovariectomized rat uteri was also investigated.

Materials and methods

Animals and housing conditions

236 female Crj:Donryu rats at 8 weeks of age were purchased from Charles River Japan (Kanagawa, Japan). The animals were maintained in an air-conditioned animal room under constant conditions of 24 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle (light, 08:00–20:00; dark, 20:00–08:00), housed three or four to a cage. Commercial powder diet (CRF-1, Oriental Yeast, Kanagawa, Japan) and drinking water were available *ad libitum* for the acclimatizing period. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals

I3C, 4HE, 2HE and 16α-hyroxyestradiol (16αHE) were purchased from Sigma-Aldrich (MO), *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG) from Nacalai Tesque (Kyoto, Japan), and E2 and dimethylsulfoxide (DMSO) from Wako Pure Chemicals (Osaka, Japan).

Selection of dosing of I3C

2000 p.p.m. of I3C in diet has been reported as an effective dose in a multi-organ tumorigenesis model in rats (12) and 500 p.p.m. is known to induce cytochrome P450s in the rat liver (20).

Uterotrophic assay (experiment 1)

To assess estrogenic or anti-estrogenic activity of I3C, 38 female Donryu rats were ovariectomized under ether anaesthesia at 9 weeks of age, and starting 2 weeks thereafter were assigned to nine groups receiving: only ovariectomy (controls); daily s.c. treatment of E2 at a dose of 1 μg/kg; 4HE at a dose of 5 μg/kg; 2HE at a dose of 5 μg/kg; 16αHE at dose of 1 μg/kg: daily administration of 500 or 2000 p.p.m.-I3C in basal diets (I3C500 or I3C2000, respectively); or daily s.c. treatment of 1 mg/kg E2 plus I3C500 or I3C2000 for 2 weeks. After 2 weeks treatment, all animals were killed and the uteri were weighed. The uteri and livers were fixed in 10% neutral-buffered formaldehyde solution, routinely processed, sectioned and stained with hematoxylin and eosin. The uteri were measured for the height of the luminal epithelium with an image analyzer, IPAP-Win (Sumika-techno Service Co., Osaka, Japan).

Uterine carcinogenesis (experiments 2 and 3)

To clarify the effects of I3C on rat uterine endometrial adenocarcinoma development, female rats were treated with a single dose of 20 mg/kg ENNG into a unilateral uterine horn via the vagina using a stainless catheter at 11 weeks of age. This is known to exert no carcinogenic effects except in the uteri (39). After the initiation, in experiment 2, 30 animals were fed dietary I3C500 up to 15 months of age (for 12 months), and compared with 24 control rats fed powder basal diet without I3C. At 15 months of age, all surviving animals were necropsied (experiment 2).

For experiment 3 to elucidate sequential changes regarding the effects of I3C on uterine carcinogenesis and hepatic metabolism of E2, 144 females were allocated to the following four groups after the ENNG initiation: control (39 females); dietary I3C2000 (39 females); and twice weekly s.c. treatment with 1 μg/kg E2 (E2, 30 females) or 5 μg/kg 4HE (4HE, 36 females). At 6, 9 and 12 months of age, four to nine animals per group were examined, and all survivors were terminated at 15 months of age. After macroscopic examination, the reproductive system and related organs, including the ovaries, uteri and vagina, endocrine system organs and any macroscopical abnormalities, were fixed in 80% cold ethanol solution (uteri), or 10% neutral-buffered formaldehyde solution (other organs). These tissues and/or organs fixed were routinely processed for histopathological examination.

In both experiments, the upper, middle and lower parts of each uterine horn and the cervix were cut into three pieces in cross-section to evaluate uterine proliferative lesions, classified into three degrees of atypical hyperplasia (slight, moderate or severe) and adenocarcinomas, according to the criteria described previously (37,38). Briefly, slight hyperplasia was used when the numbers of glands with no or slight cellular atypia were increased within the endometrium. Moderate hyperplasia referred to increased numbers of glands with slightly to moderately atypical cells in focal and/or diffuse areas of the endometrium. Severe hyperplasia was composed of irregular proliferations of atypical glands in diffuse area of the endometrium. Adenocarcinomas were diagnosed on the basis of invasion of tumor cells into the muscularis. In addition, adenocarcinomas were subdivided into well, moderately and poorly differentiated types, and also classified as to the degree of invasion: limited to the uterus, invading into the serosa and/or surrounding adnexae, and with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers (43). Animals found dead or killed when moribund were also examined in the same manner. Throughout the two experiments, body weights were measured at regular intervals and clinical signs were checked daily for all animals.

Estrous cyclicity

Vaginal cytology was observed in all animals to investigate estrous cyclicity throughout the study (experiments 2 and 3).

Preparation of livers

At 6, 9, 12 and 15 months of age in experiment 3, right and median lobes of selected livers of each group were frozen in liquid nitrogen for analysis of enzyme activities related to estrogen metabolism (6, 9, 12 and 15 months of age) or mRNA expression of cytochrome P450s by reverse transcription PCR (RT–PCR) (15 months of age), and stored at −80°C until use.

mRNA expression of cytochrome P450 enzymes in the liver

Small pieces of the liver (~200 mg) were obtained from three control and four I3C2000-treated animals in experiment 3 at 15 months of age. The samples were homogenized in 4 ml RLT buffer, mixed with 40 μl β-mercaptoethanol, and RNA was isolated using an RNeasy Midi extraction Kit (QIAGEN, Germany) and stored at −80°C until RT–PCR analysis.

RT–PCR and PCR primers of cytochrome P450 1A1, 1A2, 1B1 and GAPDH mRNA transcription in the present study were done as reported previously (26,44,45). Aliquots (500 ng) of total liver RNA were used for the RT–PCR. The primers were synthesized and purified by Takara Bio (Shiga, Japan). Levels of cytochrome P450s mRNA expression relative to GAPDH mRNA expression were calculated as ratios using an image analyzer (NIH image, Bethesda, MD).

Immunohistochemical distribution of cytochrome P450 enzymes in the liver

Cytochrome P450 protein amounts in the liver were examined immunohistochemically using paraffin-embedded sections from animals in experiments 2 and 3. After blocking endogenous peroxidase by incubation with hydrogen peroxidase (3%, v/v) in methanol, deparaffinized liver sections were incubated with anti-rat CYP 1A1, 1A2, 2B1 or 3A2 (Daiichi Pure Chemicals, Tokyo, Japan), diluted 1:100 in Tris-buffered solution (Takara Bio) with 1% skim milk at 37°C for 1 h. After the incubation, the sections were exposed to secondary antibodies and linked with streptavidin peroxidase using a DAKO LSAB+ kit (DAKO cytomation, CA). Binding was visualized by incubating sections with 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals), and counterstaining with hematoxylin for histopathological examination. Immunohistochemical distribution of CYP IB1 could not be examined in the present study, because no anti-rat CYP 1B1 antibody was available for immunohistochemistry using formalin-fixed and paraffin-embedded sections.

Enzyme activities related to estrogen metabolism in the liver

Estradiol 2- and 4-hydroxylase and 16α-hydroxylase activities in liver (median lobe) samples obtained from four or five rats in the control-, I3C2000-, E2- or 4HE-treated groups at 6, 9, 12 and 15 (except 16α-hydroxylase activity) months of age in experiment 3 were determined by SRL (Tokyo, Japan), as for previous reports (7,22).

Statistical analysis

Values for incidences including data of uterine proliferative lesions and estrous cyclicity were analyzed statistically using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between the treated and control groups were made with the Dunnett's *t*-test. *P* values < 0.05 were considered to be statistically significant. In the uterotrophic assay, the uterine weights and heights in treated groups were compared with those in the control (only ovarietomized rats) and positive control (E2-treated) groups.

Results

Estrogenic or anti-estrogenic activities of I3C (experiment 1)

Uterine weights and heights of the luminal epithelium are shown in Figure 1. Neither dose of I3C affected parameter in ovariectomized rats, with or without E2 replacement. The uterine weights and heights with 5 μg/kg 4HE treatment were comparable with those with 1 μg/kg E2 treatment, while 16αHE and 2HE treatments had much lower and no estrogenic activity, respectively.

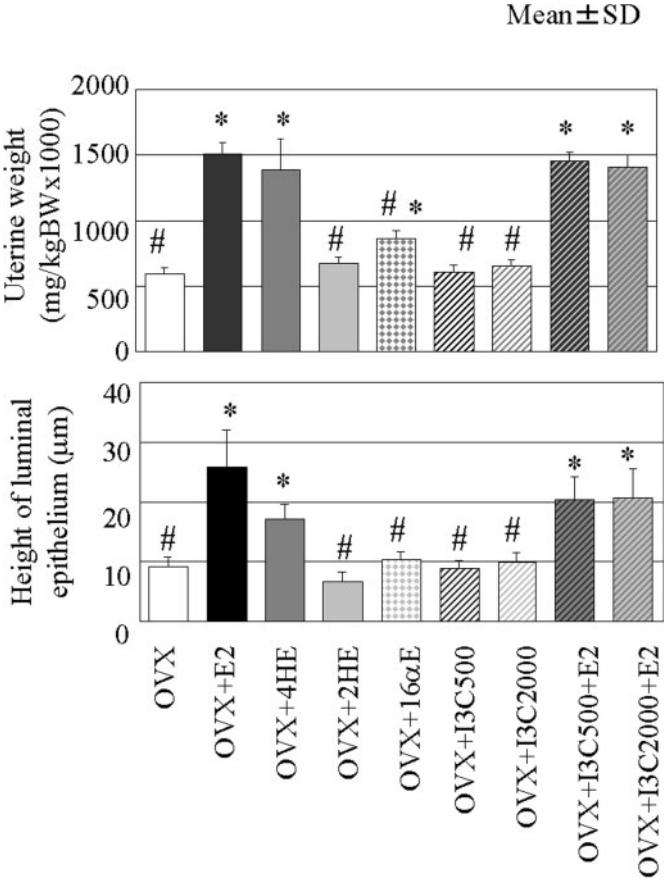

Fig. 1. Relative uterine weights and heights of uterine luminal epithelium in experiment 1. **P* or #*P* refer to significant differences from the control (ovariectomy only) and E2-treated groups, respectively, at 1% or below.

Body weights, clinical signs, survival curves and estrous cyclicity (experiments 2 and 3)

Body weights were depressed by I3C treatment with both doses (data not shown). During experiments 2 and 3, no treatment-related clinical signs were observed and survival curves in all treated groups were comparable with those of the relevant control groups (data not shown). In both experiments, I3C and E2 treatment did not increase persistent estrus (PE) status up to 15 months of age, while subcutaneous treatment of 4HE in experiment 3 significantly increased PE status after 5 months of age (Figure 2).

Effects of I3C on uterine carcinogenesis (experiments 2 and 3)

Incidences of uterine proliferative lesions and data for their multiplicity are shown in Table I. In experiment 2, the incidence of adenocarcinomas in the group treated with I3C500 was significantly elevated compared with the control group. In experiment 3, the incidence of adenocarcinomas was significantly increased in the 4HE group, compared with that of the control group. I3C2000 and E2 treatments also increased the incidences (44 and 50%, respectively) as compared with the control value (22%), but not significantly. Multiplicities of the uterine proliferative lesions were significantly increased by both I3C and 4HE treatments, whereas only a tendency for increase was evident with E2. Histologically, almost all uterine adenocarcinomas were of well-differentiated type, and morphological or biological malignancy was not influenced by the I3C treatment. In sequential observation of uterine tumor development, slight atypical hyperplasias had already appeared in the 4HE- and E2-treated groups at 6 months of age when no proliferative lesions were detected in controls. Development profiles for uterine proliferative lesions for I3C-treated and control animals were comparable up to 12 months of age.

Pathological examination of other organs

At all examined times in experiment 3, the relative liver weights were consistently elevated in the I3C2000 treated group (data no shown). Microscopically, centrilobular hypertrophy of hepatocytes was observed in all I3C-treated groups of experiments 1, 2 and 3. Most ovaries in all groups were atrophic with small cystic atretic follicles and lacking corpus lutea at termination of experiments 2 and 3. In these two experiments, various non-neoplastic and neoplastic lesions were observed in representative organs and other endocrine tissues; however, all lesions were similar to those detected

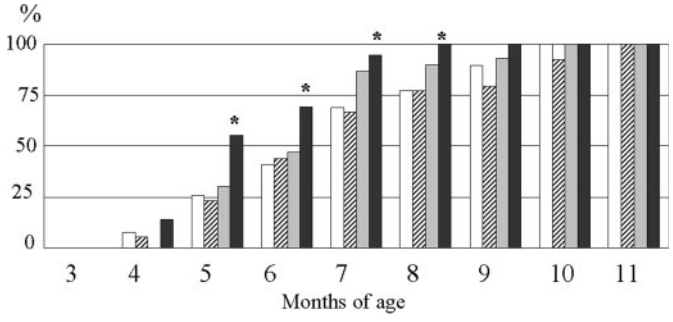

Fig. 2. Percentage incidences of animals showing PE from vaginal cytology in experiment 3. White, stripe, gray and black columns indicate the control, I3C2000-, E2- and 4HE-treated groups, respectively. **P* refers to significant differences from control incidences at 5% or below.

Table I. Incidence of uterine proliferative lesions[a] and their multiplicities in experiments 2 and 3

	No. of rats with no abnormalities	Hyperplasia			Adenocarcinoma	Multiplicities[b]
		Slight	Moderate	Severe		
Experiment 2						
15 months of age						
Control ($n = 24$)	4	2	5	7	6	1.04 ± 0.62
I3C500 ($n = 30$)	1	2	3	7	17*	1.50 ± 0.63*
Experiment 3						
15 months of age						
Control ($n = 18$)	2	2	7	3	4	1.17 ± 0.62
I3C2000 ($n = 18$)	1	2	5	2	8	1.78 ± 0.73**
E2 ($n = 16$)	0	3	2	3	8	1.50 ± 0.52
4HE ($n = 16$)	0	0	5	1	10*	1.69 ± 0.60**

[a]Uterine proliferating lesions include slight to severe atypical hyperplasia and adenocarcinomas, these criteria referred to Nagaoka *et al*. (37,38).
[b]Multiplicities are calculated average number of uterine proliferative lesion per rats, and indicated mean ± SD.
Values in parentheses show the number of rats examined.
*,**Significantly different from relevant control group at $P < 0.05$ and $P < 0.01$, respectively.

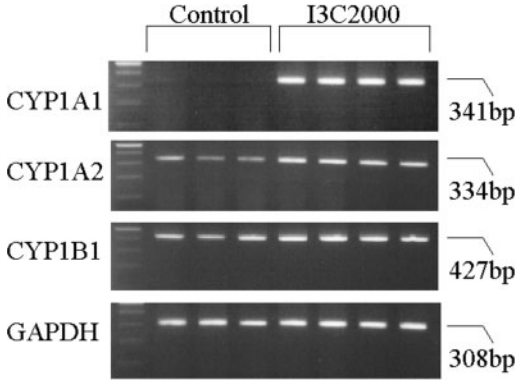

Fig. 3. mRNA expression for CYPs 1A1, 1A2, 1B1 and GAPDH in the livers of control and I3C2000-treated groups at 15 months of age in experiment 3.

spontaneously in this rat strain (36), and there were no differences in these lesions among the groups. Necropsy of animals found dead or killed when moribund also did not reveal any treatment-related changes.

mRNA expression of cytochrome P450s in the liver

Findings for mRNA expression of CYP 1A1, 1A2 and 1B1 in the liver of experiment 3 are demonstrated in Figures 3 and 4. In the control group, CYP 1A1 was not detectable. I3C treatment significantly increased CYP 1A1, 1A2 and 1B1 mRNA expression compared with the control group, with induction of 1A1 expression being the most prominent.

Immunohistochemical staining of cytochrome P450s

CYP 1A1 and 1A2 were clearly demonstrable in the hepatocytes of centrilobular areas in all I3C-treated groups in experiments 2 and 3 up to 12 months of age, while very weak expression of 1A2 was observed in relevant controls (Figure 5). At 15 months of age, 1A1 expression in the I3C-treated group was similar to that at the other examined times, whereas 1A2 expression was too varied to detect any differences from relevant controls in experiments 2 and 3. Results for other CYPs such as 2B1 or 3A2 were comparable among the livers in the control and treated groups up to 15 months of age.

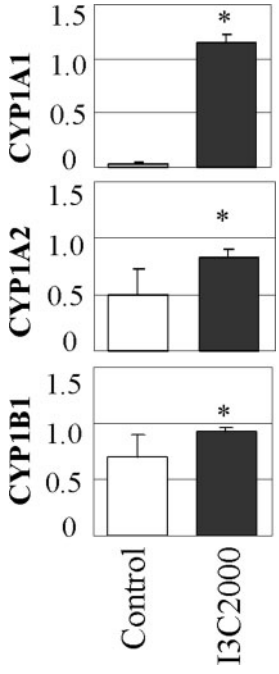

Fig. 4. Levels of expression of CYPs 1A1, 1A2 and 1B1 mRNAs relative to GAPDH mRNA in the liver, as for Figure 3. The intensities of P450s are relative to GAPDH mRNA levels (calculated as 1.0).

Enzyme activities related to estrogen metabolism in the liver

Estadiol 2-, 4- and 16α-hydroxylase activities in the liver (experiment 3) are shown in Table II. The estradiol 2-hydroxylase activities in the I3C-, E2- and 4HE-treated groups showed increasing trends compared with the control group at most of the examined points. However, there were no significant differences among them due to great variation except 15 months of age, when a significant increase was increased by I3C treatment. The 4-hydroxylase activities demonstrated significant increases in the I3C- and 4HE-treated groups at 9 and 15 months of age, or tendencies for increase in all treated groups at all examined times, except the 4HE-treated group at 12 months of age. At all examined points, 16α-hydroxylase activities showed neither significant differences nor any tendency for change with the treatments.

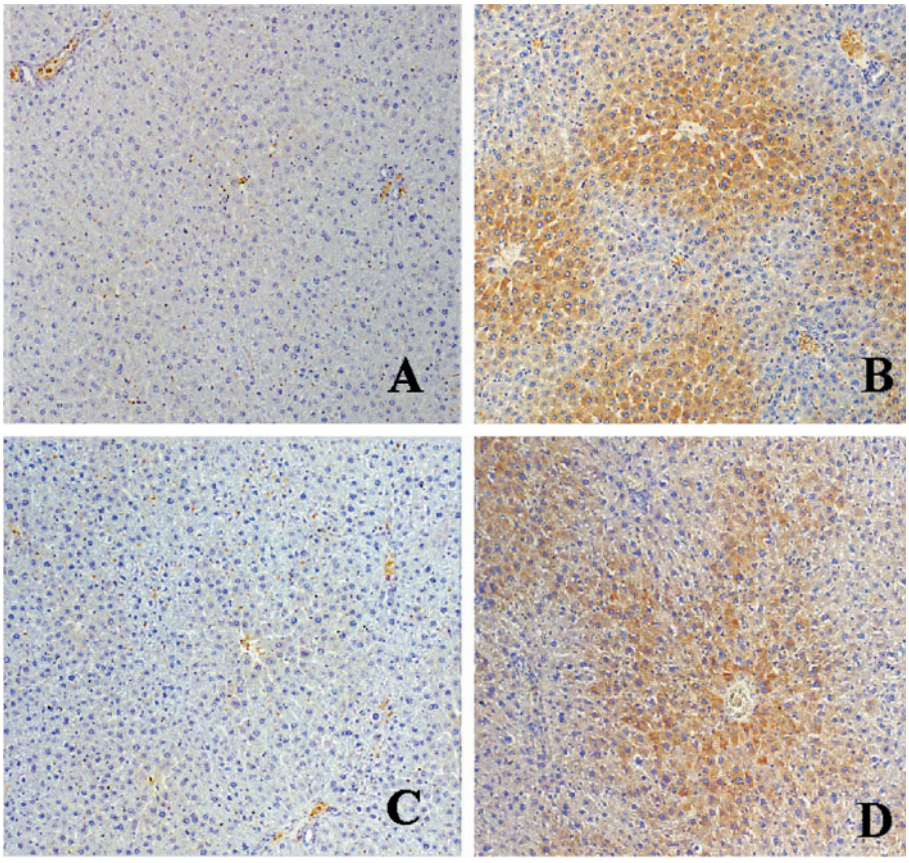

Fig. 5. Immunohistochemical staining of CYP 1A1 and 1A2 in the livers of animals at 15 months of age in experiment 3. (**A** and **B**) CYP 1A1 expression in control and I3C2000-treated animals. (**C** and **D**) CYP 1A2 expression in control and I3C2000-treated animals. Hematoxylin was used for counterstaining.

Table II. Enzyme activity related to estrogen metabolism in the liver (experiment 3)

	Enzyme activities related to estrogen metabolism (pmol/min/mg protein)		
	Estradiol 2-Hydroxylase	Estradiol 4-Hydroxylase	Estradiol 16α-Hydroxylase
6 months of age			
Control (5)	66.76 ± 23.50	2.97 ± 1.18	2.88 ± 1.00
I3C2000 (5)	139.72 ± 83.72	5.24 ± 2.39	2.65 ± 1.31
E2 (3)	104.88 ± 23.44	5.46 ± 2.40	3.34 ± 0.59
4HE (4)	108.14 ± 5.84	5.66 ± 0.58	4.37 ± 0.90
9 months of age			
Control (5)	61.82 ± 29.35	1.78 ± 0.73	1.30 ± 0.21
I3C2000 (5)	88.30 ± 25.79	4.19 ± 1.63*	1.75 ± 0.62
E2 (4)	92.72 ± 24.47	3.44 ± 1.66	2.18 ± 0.34
4HE (3)	110.22 ± 31.36	4.60 ± 1.54*	1.99 ± 0.85
12 months of age			
Control (4)	78.05 ± 29.45	3.52 ± 2.52	1.24 ± 0.36
I3C2000 (4)	149.80 ± 51.88	5.14 ± 2.52	1.05 ± 0.13
E2 (4)	86.37 ± 64.90	5.06 ± 3.16	1.47 ± 0.64
4HE (4)	78.57 ± 8.63	3.20 ± 0.49	1.42 ± 0.64
15 months of age			
Control (4)	75.08 ± 15.23	3.94 ± 1.18	NE
I3C2000 (4)	205.32 ± 51.92**	7.14 ± 1.36**	NE
E2 (4)	67.79 ± 10.55	3.01 ± 0.87	NE
4HE (4)	108.16 ± 7.32	5.97 ± 0.75*	NE

Values in parentheses mean number of rats examined. Values mean average ± SD. NE, not examined.
*Significantly different from control group, $P < 0.05$.
**Significantly different from control group, $P < 0.01$.

Discussion

In the present study using rat uterine cancer model, dietary treatment with I3C clearly demonstrated promoting effects on endometrial adenocarcinoma development. I3C can act both as an inhibitor and promoter of carcinogenesis, and our data are in line with the promoting results observed earlier with several animal carcinogenesis models (11,12). As for a cause of the complex effects, I3C is unstable under the acid condition and a number of acid-catalyzed metabolites such as 3,3′-diindolylmethane and indolcarbazole are produced in the gut (46). The acid condensation product has shown to be a potent aryl hydrocarbon receptor agonist, providing anti-estrogenic and antitumorigenic activity (47). In the present study, the activity of each acid-catalyzed metabolite of I3C to the rat uteri was not investigated; however, the dietary treatment with I3C at doses of 500 or 2000 p.p.m. did not show any estrogenic- or anti-estrogenic activity in the rat uteri, indicating that the promoting effect did not result from direct binding of I3C to estrogen receptor α in the rat uteri as estrogenic or anti-estrogenic agents.

I3C is widely accepted to induce CYPs 1A1, 1A2 and/or 1B1 in the liver and other organs (13–15,20,21). In rats, CYPs 1A1 and 1A2 catalyze mainly E2 into 2HE, the dominant product of catechol estrogen with weak hormonal potency and no carcinogenic effects (7,22,24,25,31), by hepatic 2-hydroxylation of estradiol (24,25), whereas CYP 1B1 is a major catalyzing enzyme of E2 to 4HE, a strongly carcinogenic and toxic metabolite (23,26,32,33,48).

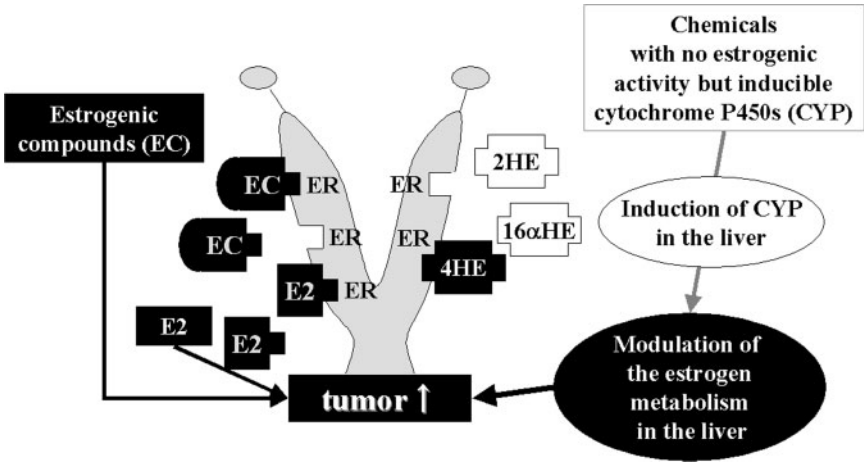

Fig. 6. Hypothesized mechanisms of promoting effects of chemicals with or without estrogenic activity on uterine carcinogenesis in rats. 16αHE, 16α-hydroxyestradiol; ER, estrogen receptor α. Black blocks indicate promotion of uterine carcinogenesis, while white blocks indicate weak or no promoting activity.

In the present study, dietary I3C treatment increased the induction of CYPs 1A1, 1A2 and 1B1 enzymes in the liver at either the mRNA level or its producing proteins. In the assays of estradiol hydroxylase activities in the liver, dietary I3C increased both 2- and 4-hydroxylase activities, in particular the latter. These results strongly suggest that the induction of the CYP 1 family by I3C is linked to modulation of E2 metabolism. In this study we could not determine which enzyme in the CYP 1 family was most effective in this regard. The present finding that 4HE treatment increased uterine adenocarcinoma development provides the evidence that it possesses stronger carcinogenic effects on rat uterus than E2, whereas uterotrophic activity of 4HE was weaker, in line with previous reports (23,32,33,48).

Endometrial adenocarcinoma development is strongly related to estrogen exposure in women and the Donryu rat features endocrinological similarities to the human case, ovarian hormonal imbalance leading to elevation of the serum estrogen/progesterone ratio, manifested as atrophic ovary with small polycystic atretic follicles and lack of corpora lutea and a long-term PE status as indicated by vaginal cytology (36–38). Using the two-stage uterine carcinogenesis model in this rat strain (39), continuous stimulation by estrogens or estrogenic compounds, which directly bind to estrogen receptor in the uteri or induction of early occurrence of the PE status enhanced uterine carcinogenesis (49,50).

In the present study, dietary I3C enhanced uterine carcinogenesis without affecting estrous cyclicity or showing estrogenic activity in the uteri. Induction of CYPs 1A1, 1A2 and 1B1 in the liver by dietary I3C and sequential modulation of estrogen metabolism therefore should be nominated as crucial to the promoting effects. The modulation, in particular the continuous increase of 4HE level, by I3C treatment might be important as part of the hypothesized pathway described schematically in Figure 6.

A number of chemicals and environmental pollutants induce CYP 1 family enzymes in the liver or other organs (26,34,51,52). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin or diesel exhaust is reported to up-regulate CYP 1B1 (45,51–54). In most mammalian species, the main estradiol metabolites were generated by hepatic microsomal P450s in the liver and other tissues (22,23) and the functions and regulation of CYPs 1A1 and 1A2 appear to be highly conserved (55). Therefore, animal data concerning modulation of estrogen metabolism via induction of CYP 1 family may provide useful information for human risk assessment, although further investigations are required to detail their exact significance.

The precise reason for the discrepancy between the promotion observed here and the previous report that dietary I3C inhibited spontaneous uterine tumor development in Donryu rats (7) could not be determined. The differences might be due to the dietary doses applied, or resultant variation in the ratios of E2 to 2HE and 4HE, especially the latter, in addition to the difference in the uteri with or without initiation of ENNG. Several reports proposed that the ratio of 2HE/4HE formation was important as a marker of estrogen-dependent tumor development (26,33).

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