Jpn. J. Cancer Res. **90**, 399–405, April 1999

Organ-dependent Modifying Effects of Caffeine, and Two Naturally Occurring Antioxidants α-Tocopherol and *n*-Tritriacontane-16,18-dione, on 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced Mammary and Colonic Carcinogenesis in Female F344 Rats

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Modifying effects of caffeine, α-tocopherol, and *n*-tritriacontane-16,18-dione (TTAD) on 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced mammary and colonic carcinogenesis were investigated in female F344 rats. Groups of 20 rats, 6 weeks old, were given 0.02% PhIP (in diet) alone, or together with 0.1% caffeine (in drinking water), 0.5% α-tocopherol (in diet) or 0.1% TTAD (in diet) for up to 54 weeks. Groups of 10 females receiving basal diet or one of the test chemicals without PhIP supplementation were also maintained. The final combined incidences (adenomas plus adenocarcinomas) and multiplicity (No./rat) of mammary adenomas and adenocarcinomas were significantly lowered in the PhIP plus caffeine group (10%, 0.10) as compared to the PhIP alone value (40%, 0.50). Incidences of mammary tumors in the PhIP plus α-tocopherol or TTAD groups tended to be decreased while their multiplicities were significantly lowered. With regard to colon tumor development, on the other hand, rats given PhIP plus caffeine exhibited an elevated incidence (75% versus 15% in the control), whereas α-tocopherol and TTAD had no effect. Surprisingly, metabolic activation of PhIP was inhibited by addition of caffeine in an *in vitro* assay. The results indicate that caffeine exerts a potent chemopreventive action against PhIP-induced mammary carcinogenesis, but acts as a co-carcinogen for PhIP-induced colonic carcinogenesis.

Key words: Chemoprevention — Co-carcinogen — PhIP — Caffeine — Mammary and colonic carcinogenesis

Among the around 20 heterocyclic amines found in cooked meat and fish, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) has been identified as the most abundant.[1–3] It has been demonstrated to possess mammary, colon and prostate carcinogenic potential in rodents.[4-7] As the risk of mammary and colon tumors is quite high in the populations of Western countries where large amounts of meat are consumed, this food-derived carcinogen could be of great importance as an environmental factor. It might thus be possible to prevent cancer by reducing the exposure to heterocyclic amines through a change in life style. Another approach is to find chemopreventive agents, which modify metabolic activation or detoxification of this food-derived carcinogen. PhIP is known to undergo metabolic activation by *CYP1A2* or *CYP1B1* in the liver[8–12] and partially in the target organs.[13, 14] Recently, several candidates for chemoprevention have been investigated using this environmental carcinogen,[15–18] and antioxidants, particularly 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ)[16] may exert potent inhibiting effects against PhIP-induced mammary carcinogenesis.

Caffeine has been extensively studied epidemiologically and experimentally for risk assessment in view of the large amounts consumed.[19] With initiation/promotion protocols using mammary gland specific chemical carcinogens such as *N*-methyl-*N*-nitrosourea (MNU) and dimethylbenz[*a*]anthracene (DMBA),[20, 21] no inhibiting effects were reported. However, caffeine is a CYP1A2 inducer,[9] and therefore might be expected to modify PhIP carcinogenesis when given concomitantly.

α-Tocopherol is a lipophilic antioxidant with similar chemical structure to the chemopreventive agent HTHQ. It has been shown to inhibit Glu-P-1-induced hepatocarcinogenesis,[22] but not DMBA-induced mammary tumor

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development.[20, 23, 24] However, α-tocopherol acts as an enhancer of the chemopreventing effects of selenium on rat DMBA-induced mammary tumor carcinogenesis.[20]

n-Tritriacontane-16,18-dione (TTAD), a β-diketone derivative, is contained in *Eucalyptus* leaf wax.[25, 26] Since β-diketones have been shown to reduce mutagenicity and nucleic acid binding of 2-aminofluorene derivatives[27] and TTAD inhibited hepatic and pancreatic carcinogenesis in a medium-term multi-organ bioassay,[28] it was also included for investigation in the present study of modifying potential in a PhIP-induced mammary and colon carcinogenesis model.

MATERIALS AND METHODS

Chemicals PhIP (Lot. No. 891110) was obtained from NARD Institute, Ltd., Osaka, caffeine anhydrous (Lot. No. DSM 5327) from Wako Pure Chemical Industries, Ltd., Osaka, and α-tocopherol (Lot. No. 01060601) from Eisai Co., Ltd., Tokyo. TTAD was a gift from Eisai Co., Ltd., Tsukuba.

Animals and maintenance A total of 120 F344/DuCrj female rats, obtained from Charles River Japan Inc. (Atsugi), were used in the study. The rats were about 6 weeks old at the commencement and were housed 5 to a plastic cage on hardwood chip bedding (Beta chip, Northeastern Products Co., New York, NY) in an environment-controlled room. Constant conditions of temperature (22±2°C), humidity (55±10%), and ventilation (more than 15 times/h all fresh system) were maintained, and the room was artificially illuminated for 12 h each day.

Experimental procedures

Protocol: The animals were divided into 8 groups using a computerized stratified body weight technique, so that the weight distribution within each group was similar. Starting at the age of 6 weeks, 4 groups of 20 rats each received powdered basal diet MF (Oriental Yeast Co., Ltd., Tokyo) containing 0.02% PhIP either alone or in combination with α-tocopherol 0.5% diet, TTAD 0.1% diet, or caffeine 0.1% drinking water. The doses were chosen on the basis of earlier study results.[15–18, 20, 21, 23, 24, 28] Further groups of 10 rats each were given test chemicals or basal diet alone without PhIP. Diet preparation was performed at monthly intervals, with storage in the dark at room temperature. Drinking water was freshly prepared every 2 or 3 days.

Antemortem examination: The animals were observed daily for clinical signs and mortality, and carefully palpated biweekly for the presence of mammary tumors. Data on size, location, and numbers of tumors were recorded. Individual body weights were measured weekly for the first 4 weeks and every other week thereafter. Food and water consumption was measured over a 2-day period before each weighing.

Postmortem examination: Moribund rats were killed for autopsy in the course of the study, and all surviving animals were killed after week 54. Whole skin with mammary glands and tumors, as well as the heart, lung, stomach, intestine, liver, kidneys and urinary bladder were removed and fixed in 10% buffered formalin solution. Histopathological examination was performed on hematoxylin and eosin-stained tissue sections.

Analysis of caffeine effects on the metabolic activation of PhIP Rat S9 mixture (+NADPH 4.5 m*M*) and caffeine (0.1, 0.3, 1.5 and 3.0 m*M*) were preincubated for 2 min at 37°C and then mixed with 0.1 m*M* PhIP or Glu-P-1 as previously reported.[29] After incubation for 20 min at 37°C metabolites were extracted with ice-cold methanol (PhIP) or acetonitrile (Glu-P-1) and centrifuged for 5 min at 10,000*g*. Supernatant samples were analyzed by high-performance liquid chromatography (HPLC) (Hitachi 1-6200 intelligent pump, Hitachi Co., Ltd.) under the following conditions: PhIP mobile phase; methanol/water (0.1% DEA, pH 7.0), 41/59; flow rate, 1.0 ml/min; column, COSMOSIL 5C18, 4.6×150.0 mm; detection, ultraviolet light (UV) at 315 nm (UV detector, Hitachi S-1050), Glu-P-1 mobile phase; acetonitrile/20 m*M* $NH_4H_2PO_4$ (pH 4.0); flow rate, 1.0 ml/min; column, COSMOSIL 5C18, 4.6×150.0 mm; detection, fluorescence at E_x D265 nm and E_m 403 nm (fluorescence detector, Hitachi L-4250).

Statistical analysis The significances of intergroup differences in numerical data obtained for body weights, organ weights and enzyme-altered foci were assessed using the two-sided Student's *t* test. Insufficient homogeneity of variance was corrected with respect to the degree of freedom according to the method of Welch.[30]

RESULTS

In the present study, none of the rats exposed to test chemicals showed any clinical signs until development of palpable subcutaneous mammary tumors was noted from week 32, limited to the PhIP-treated groups. Results for sequential observation of mammary tumors by palpation are presented in Fig. 1. In the PhIP alone group 1, the first tumor appeared at week 32 and the cumulative incidence gradually increased until finally 35% of the rats had mammary tumors. The incidences tended to be lowered in groups 2–4, and especially in group 2 (PhIP+caffeine), in which a palpable tumor was detected in only one animal during the course of the study. An animal in group 1 (PhIP alone) died of mammary tumor development at week 45, and the final survival rates in groups 1–4 were 85, 95, 90, and 90%, respectively. Average food and water consumption tended to be increased in rats given 0.1% caffeine in the drinking water, and PhIP intake in this group also tended to be increased (Table I). The average

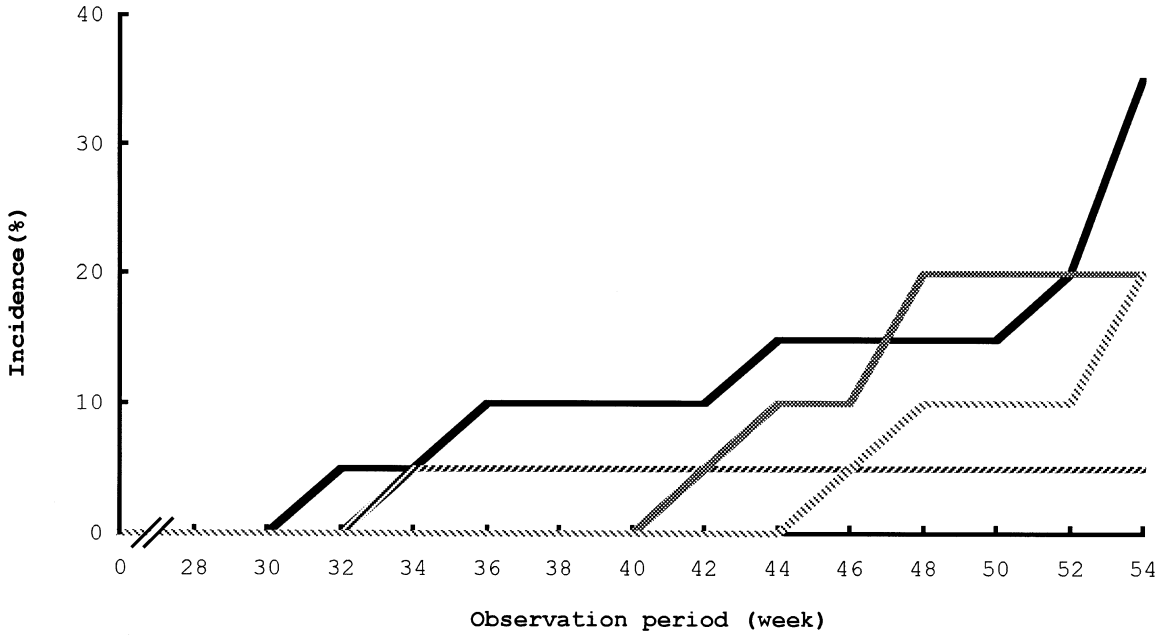

Fig. 1. Sequential observation of palpable mammary nodules/masses in female F344 rats treated with 0.02% PhIP alone or simultaneously with 0.1% caffeine, 0.5% α-tocopherol or 0.1% TTAD for up to 54 weeks. Data from non PhIP-treated groups were excluded. ▬ group 1 (PhIP alone), ▨ group 2 (PhIP+caffeine), ▩ group 3 (PhIP+α-tocopherol), ▧ group 4 (PhIP+TTAD).

Table I. Average Food Consumption, Water Consumption, and Chemical Intake Data for Rats Exposed to PhIP and Test Chemicals for 54 Weeks

Group	Treatment[a,b]	Average food consumption (g/rat/day)	Average water consumption (g/rat/day)	Average chemical intake	
				PhIP (mg/kg/day)	Test chemical (mg/kg/day)
1	PhIP (0.02)	9.9	13.8	10.6	—
2	PhIP (0.02)+caffeine (0.1)	11.0	16.1	11.9	86.9
3	PhIP (0.02)+α-tocopherol (0.5)	9.6	14.0	10.2	254.6
4	PhIP (0.02)+TTAD (0.1)	9.7	13.2	10.8	54.2

a) Numbers in parentheses are dietary levels (%).
b) Data from non PhIP-treated groups were excluded.

caffeine, α-tocopherol and TTAD intakes calculated from the nominal concentration, the mean food/water consumption and the mean body weights were 86.9, 254.6 and 54.2 mg/kg body weight/day, respectively.

The final body weights in the group treated with PhIP plus TTAD (group 4) were lower than for animals receiving PhIP alone (Table II). The relative liver weights of rats treated with PhIP plus TTAD were significantly higher than the control group (PhIP alone) value. The relative kidney weights of rats given PhIP plus α-tocopherol (group 3) were significantly lowered.

The incidences and multiplicities of mammary tumors at the end of the experiment are shown in Table III. Values for group 2 (PhIP+caffeine) were significantly lower than for PhIP alone controls. The incidences of mammary tumors in groups 3 and 4 (PhIP+α-tocopherol/TTAD) also tended to be lowered, and the multiplicities significantly decreased. Mammary tumor distribution (left/right side of cervical-thoracic, and abdominal-inguinal regions) appeared random in each group.

The incidences of adenomas and adenocarcinomas in the colon were significantly elevated in group 2 (PhIP+caffeine) as compared to the control (group 1; PhIP alone) values. In contrast, α-tocopherol and TTAD did not affect the induction of colonic tumors. Neoplastic lesions in group 2 mainly developed in the distal colon.

Table II. Final Body and Relative Organ Weight Data for Rats Exposed to PhIP and Test Chemicals for 54 Weeks

Group	Treatment[a,b]	No. of rats	Final body weight(g)	Organ-to-body weight ratio(%)	
				Liver	Kidneys
1	PhIP (0.02)	17	201.0±7.8	2.24±0.66	0.72±0.06
2	PhIP (0.02)+caffeine (0.1)	19	197.9±8.7	2.38±0.11	0.75±0.04
3	PhIP (0.02)+α-tocopherol (0.5)	18	206.2±10.3	2.27±0.15	0.68±0.04**
4	PhIP (0.02)+TTAD (0.1)	18	189.4±6.3**	2.94±0.43**	0.75±0.04

a) Numbers in parentheses are dietary levels (%).
b) Data from non PhIP-treated groups were excluded.
** $P<0.01$.

Table III. Histopathology of the Mammary Glands and Large Intestines of Rats Exposed to PhIP and Test Chemical for 54 Weeks

Site and type of neoplastic lesion[a]		Group 1 (PhIP alone)	Group 2 (PhIP+caffeine)	Group 3 (PhIP+α-tocopherol)	Group 4 (PhIP+TTAD)
Mammary gland	No. examined:	20	20	20	20
Tumor		8 (40)[c]	2 (10)*	4 (20)	3 (15)
Adenoma		1 (5)	1 (5)	1 (5)	1 (5)
Adenocarcinoma		8 (40)	1 (5)**	3 (15)	2 (10)
Multiplicity mammary tumors (No./rat)[b]		0.50±0.51	0.10±0.31**	0.20±0.41*	0.15±0.37*
Colon	No. examined:	20	20	20	20
Tumor		3 (15)	15 (75)**	1 (5)	3 (15)
Adenoma		1 (5)	8 (40)**	0	0
Adenocarcinoma		2 (10)	10 (50)**	1 (5)	3 (15)
Multiplicity of colonic tumors (No./rat)[b]		0.15±0.37	0.90±0.64**	0.05±0.22	0.15±0.37

a) Data from non PhIP-treated groups were excluded, since no neoplastic lesions were found.
b) Mean±SD.
c) Values in parentheses are percentages.
*, ** $P<0.05$, 0.01, respectively.

Table IV. Inhibitory Effects of Caffeine on PhIP Oxidation by a Rat S9 Mixture

Caffeine dose (m*M*)	PhIP (% of control)					Glu-P-1 (% of control)	
	Metab. A	Metab. B	Metab. C	Metab. D	Metab. E	Metab. A	Metab. B
0 (control)	100	100	100	100	100	100	100
0.1	127	96.9	112	117	115	105	116
0.3	101	74.2	103	91.7	90.2	97	97.7
1.5	62.7	40.3	53.8	63.4	43.9	103	64.7
3.0	32.3	21.8	0	39	19.8	83	31.1

Retention times of PhIP metabolites A, B, C, D and E on HPLC analysis were 5.1, 7.3, 9.9, 17.3 and 19.0 min, respectively. Retention times of Glu-P-1 metabolites A and B were 21.5 and 25.0 min, respectively.

None of the rats given test chemicals without PhIP developed mammary or colonic tumors. Histopathological assessment of major organs other than the mammary gland and colon revealed no treatment-related alterations.

The HPLC profiles indicated that the amounts of all the PhIP metabolites, including the major metabolite A were decreased by treatment with caffeine in a dose-related manner (Table IV). The Glu-P-1 profile also showed that metabolite B was inhibited by addition of caffeine, with clear dose-dependence.

DISCUSSION

The present investigation clearly confirmed the mammary and colon carcinogenicity of PhIP in female F344 rats[4, 6] and also revealed organ-specific modification

potential of caffeine. Since average food consumption in the group given PhIP simultaneously with caffeine appeared higher than the PhIP alone group, the observed inhibition of mammary carcinogenesis was not associated with a reduction of carcinogen ingestion and indeed, increased lesion development was noted in the colon.

As it was previously reported that caffeine did not influence the incidence of spontaneously occurring mammary tumors in rodents,[31, 32] it presumably exerts its effects via an impact on PhIP metabolism. In fact, caffeine at the higher doses in the present experiment clearly inhibited the metabolic activation of PhIP into proximal or ultimate carcinogenic species *in vitro*, as also observed for the novel lipophilic phenolic antioxidant HTHQ,[29] which similarly inhibits PhIP-induced mammary tumor development.[16] However, HTHQ also reduced PhIP-induced colon carcinogenesis in male rats.[33] It has been reported that aberrant crypt foci, considered to be a preneoplastic lesion, in the colon were increased in rats treated with caffeine and PhIP simultaneously in a short-term study.[34] The authors demonstrated that this could have been due to enhanced formation of electrophilic species by CYP1A2.[34] To our knowledge, however, no toxicological or morphological effects on the colon have so far been found in other experimental animals exposed to caffeine.[19, 31, 32] Furthermore, coffee consumption appears to have a protective influence against colon and rectal cancer in man, with reduction in bile acid and neutral sterol concentration in the bowel due to interference with bile secretion proposed as a possible underlying mechanism.[35] As PhIP and caffeine intakes (11.9 and 86.9 mg/kg/day, respectively) in the present study were extremely high when compared to estimated actual PhIP (mean 72, range 0–865 ng/day)[36] and caffeine (3–5 mg/kg/day)[19] ingestion levels, risk to humans might be negligible.

The present results indicated that α-tocopherol, a natural antioxidant present in various foods, such as polysaturated vegetable oils and the germ of cereal seeds, weakly inhibits induction of breast tumors by PhIP. This may be in line with the previously reported findings on spontaneous mammary neoplasia in Sprague-Dawley rats.[37] α-Tocopherol has also been shown to enhance the chemopreventive potential of selenium.[20] However, there have been several reports of no modifying effects on mammary tumor development, for example in animals treated with DMBA,[20, 23, 24, 38] or the direct carcinogen MNU.[20, 38] Whether it exerted its influence via PhIP metabolism in the present case is unclear.

TTAD similarly showed weak chemoprevention of PhIP-induced mammary carcinogenesis. It has been reported to exert an inhibitory influence on liver and pancreatic carcinogenesis,[28] presumably because of its strong antioxidant activity.[25] Some other β-diketones such as 1,1,1-trifluoroacetylacetone, acetylacetone, benzoylacetone and dibenzoylacetone, have been shown to reduce the mutagenicity of 2-nitrofluorene in the Ames assay, and 1,1,1-trifluoroacetylacetone can reduce nucleic acid binding of the carcinogen *N*-acetoxy-2-acetylaminofluorene.[27] These findings suggest that preventive effects of TTAD on PhIP carcinogenesis might be due to altered PhIP metabolism.

In conclusion, the present results indicate that caffeine may inhibit induction of mammary tumors by the environmental carcinogen PhIP, while enhancing its colon carcinogenicity. α-Tocopherol and TTAD, expected to be chemopreventive agents owing to their antioxidant activity, showed weak inhibitory effects on mammary tumor development, but not on colon carcinogenesis.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid from the Ministry of Health and Welfare for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control, Japan. The work was also supported in part by a Grant from the Society for Promotion of Pathology of Nagoya.

(Received December 4, 1998/Revised February 15, 1999/Accepted February 17, 1999)

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