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Advances in Brief

Inhibition of Lung Carcinogenesis by Black Tea in Fischer Rats Treated with a Tobacco-specific Carcinogen: Caffeine as an Important Constituent[1]

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Abstract

Here, we examined the effect of black tea and caffeine on lung tumorigenesis in F344 rats induced by the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in a 2-year bioassay. NNK was administered s.c. at a dose of 1.5 mg/kg body weight three times weekly for 20 weeks. Animals were given either black tea as drinking water at concentrations of 2%, 1%, or 0.5%, or caffeine in drinking water at concentrations identical to those in 2% and 0.5% tea infusions for 22 weeks. The treatment period began 1 week before and ended 1 week after the NNK administration. The animals were sacrificed on week 101 for the examination of tumors in target organs, including lung, liver, nasal cavity, and other major organs. The NNK-treated group, given 2% black tea, showed a significant reduction of the total lung tumor (adenomas, adenocarcinomas, and adenosquamous carcinomas) incidence from 47% to 19%, whereas the group given 1% and 0.5% black tea showed no change. The 2% tea also reduced liver tumor incidence induced by NNK from 34% in the group given only deionized water to 12%. The tumor incidence in the nasal cavity, however, was not affected by either black tea or caffeine at any of the concentrations tested. The most unexpected finding was the remarkable reduction of the lung tumor incidence, from 47% to 10%, in the group treated with 680 ppm caffeine, a concentration equivalent to that found in the 2% tea. This incidence is comparable to background levels seen in the control group. This study demonstrated for the first time in a 2-year lifetime bioassay that black tea protects against lung tumorigenesis in F344 rats, and this effect appears to be attributed, to a significant extent, to caffeine as an active ingredient of tea.

Introduction

A large body of evidence from studies in laboratory animals indicates that green tea protects against cancer development at various organ sites (1). However, the effect of black tea on carcinogenesis has been scarcely studied, although the worldwide production and consumption of black tea far exceed those of green tea (2). Previously, we have shown that green tea administered as drinking water inhibits lung tumor development in A/J mice treated with NNK,[3] a potent nicotine-derived lung carcinogen found in tobacco (3). This inhibitory effect of green tea has been attributed to its major polyphenolic compound, EGCG, and, to a lesser extent, to caffeine (4). We have also demonstrated that, although levels of O^6-methylguanine, a critical lesion in NNK lung tumorigenesis, were not affected in lung DNA, the levels of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, were significantly suppressed in mice treated with green tea and EGCG (3). These studies underscore the importance of the antioxidant activity of green tea and EGCG for the inhibitory activity against lung tumorigenesis and, together with other studies (4, 5), provide support for the hypothesis that green tea consumption may play a role in lowering the risk of lung cancer among male smokers in Japan as compared to smokers in the United States (6). These laboratory results have provided the impetus for conducting large-scale cohort or intervention studies to more closely examine the beneficial effect of green tea in protecting against human cancers.

The chemical composition of black tea differs significantly from that of green tea due to extensive oxidation of catechins during manufacturing (7). The oxidation products found in black tea, thearubigins and theaflavins, also possess antioxidant activity (8), suggesting that black tea may also inhibit NNK-induced lung tumorigenesis. Indeed, bioassays in A/J mice have shown that black tea given as drinking water retarded the development of lung cancer caused by the treatment with NNK (4, 5). However, data on the relationship of black tea consumption with the lung cancer risk in humans are limited and inconclusive (9, 10). There is a need for additional tumor bioassays in animal models to better examine the protective role of black tea against lung cancer. The development of adenocarcinomas and adenosquamous carcinomas in F344 rats upon chronic administration of NNK provides an important and relevant model for lung carcinogenesis in smokers (11). Thus far, no information was available regarding the effects of black tea and caffeine in this model. Here, we conducted a 2-year lifetime bioassay in F344 rats to determine whether black tea and caffeine are protective against lung tumorigenesis induced by NNK. We have generated important new findings that support black tea and caffeine as potential preventive agents against lung cancer and suggest that a closer examination of the roles of black tea and caffeine on lung cancer in smokers may be warranted.

Materials and Methods

Animals. Five-week-old male F344 rats were purchased from Charles River Laboratories (Kingston, NY). They were housed two or three per cage in solid-bottom polycarbonate cages with hardwood bedding and maintained under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-h light/dark cycle).

Tea Solutions and Chemicals. NNK was synthesized according to a published procedure (12). Caffeine (99% purity) was purchased from Aldrich (Milwaukee, WI). The black tea leaves were provided by the Southern Tea Company (Marietta, GA). The black tea infusions were prepared using a commercial Bunn Tea Brewer (Springfield, IL). A total of 80 g of tea leaves were extracted with 4 liters of deionized water passed from the machine through the tea leaves. The resulting solution yielded the highest test concentration (2%). Lower concentrations were prepared by appropriate dilutions of the 2% infusion, namely 1:1 dilution for 1% tea infusion and 3:1 dilutions for 0.5% tea infusion. Fresh extracts were prepared on Mondays, Wednesdays, and Fridays and used immediately for bioassay. The tea infusions were stored in the dark at room temperature. The caffeine concentration in the undiluted tea

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; EGCG, epigallocatechin gallate.

infusion (2%) was determined by a high-performance liquid chromatography method to be 0.68 mg/ml (3). All other chemicals from commercial sources were reagent grade.

Bioassay. Animals were randomly divided into six groups, as shown in Table 1. The NIH-07 diet (Dyets, Bethlehem, PA) with 5% corn oil was given throughout the bioassay. Beginning at 7 weeks of age, animals in group 1 were given only deionized water; those in groups 2a, 2b, and 2c were given tea as drinking water at 2, 1, and 0.5%, respectively; and rats in groups 3a and 3b were given water containing caffeine at concentrations of 680 ppm and 170 ppm, respectively. One week later, animals in groups 1–3 were administered NNK at a dose of 1.5 mg/kg body weight by s.c. injection three times weekly for 20 weeks or a total of 90 mg/kg body weight. Animals received tea or caffeine for 22 weeks, beginning 1 week before and ending 1 week after the NNK treatment. In groups 4 and 5, rats were given 2% tea and 680 ppm caffeine in drinking water, respectively, without carcinogen. Group 6 served as the control without any treatment. The water consumption was measured once weekly for the first 22 weeks. Body weights were recorded once weekly for the treatment period and then once monthly for the remainder of the bioassay. The bioassay was terminated during week 101; all major organs and gross lesions were harvested during necropsy and fixed in 10% buffered formalin solution. Representative sections were obtained and processed for microscopic examination. Tumor incidence was determined by dividing the number of animals with tumors by the number of animals in each group.

Statistical Methods. The survival was evaluated through life table analysis. The survival function for each group was estimated separately using the Kaplan-Meier product limit method for censored data (13). The estimated survival distributions were then compared using the log-rank test (14). The type I error rate (α) was adjusted for the specific number of pairwise comparisons made with the Bonferroni correction (15). Tumor incidence was compared among the nine groups using the χ^2 test.

Results

The body weight curves of rats in each group throughout the bioassay are shown in Fig. 1. The growth rates were comparable among all groups throughout the bioassay until the study was nearly at an end, indicating that black tea and caffeine at the dose levels examined had little adverse effect. This observation is in contrast to our previous bioassay in A/J mice, which showed a significant loss of weight gain when mice were given 2% green tea (3). In that study, the retarded weight gains were most likely caused by the caffeine in green tea because similar body weight losses were seen in groups given the same levels of caffeine in drinking water. Data on drinking water consumption for the first 22 weeks of treatment showed that rats in group 5 had the highest consumption, followed by those in group 3a, whereas group 1 had the lowest consumption. Interestingly, groups 5 and 3a were both given caffeine at the high concentration (680 ppm). The survival rates of all groups at selected intervals during the bioassay are given in Table 1. The difference in survival rates between groups 2a and 1 at week 89 was the most apparent, 35% for group 2a *versus* 72% for group 1. This difference was due to the higher mortality rate between weeks 73 and 89 in group 2a, as compared to other groups. The reason for this decrease in survival in this group is unclear. Nonetheless, there were no statistically significant differences for the overall survival rates among all groups during the bioassay. The only exception was group 5, which showed a significantly higher overall survival rate when compared to groups 1, 2a, 3a, and 3b ($P < 0.05$) and when compared to groups 2b and 2c ($P = 0.054$). After adjusting the type I error rate (α) for the number of comparisons made (in this case, 22) using the Bonferroni correction, none of these differences were significant.

The incidences of lung tumors for each group are given in Fig. 2 and Table 2. The incidence of lung tumors, including adenomas, adenocarcinomas, and adenosquamous carcinomas, in rats treated with NNK that drank only deionized water was 47% (group 1). This incidence is within the expected range based on previous bioassays (16, 17). The NNK-treated group that drank 2% black tea (group 2a) developed only a 19% lung tumor incidence ($P < 0.05$). Only one animal showed a lung adenocarcinoma in this group, whereas there were seven lung adenocarcinomas in group 1 ($P < 0.05$). Groups 2b and 2c, receiving 1% and 0.5% black tea, showed no differences in lung cancer incidence as compared to group 1. The most remarkable observation was that only 10% of the rats in group 3a, given caffeine in drinking water at a concentration identical to that found in the 2% tea infusion, developed lung tumors. This reflects an 80% reduction in tumor incidence ($P < 0.01$). The tumor incidence of this group is comparable to that of the spontaneous tumors commonly seen in the control group (11, 16, 17). Only 4 animals in group 2a developed a lung adenoma, as compared to 10 in group 1; however, this difference was not statistically significant. At the lower concentration (170 ppm), caffeine also appeared to reduce the lung tumor incidence from 47 to 20% (group 3b); this reduction in incidence, however, was not statistically different from that in group 1. Animals in groups 4, 5, and 6 developed either background incidence of lung tumors or no tumor at all.

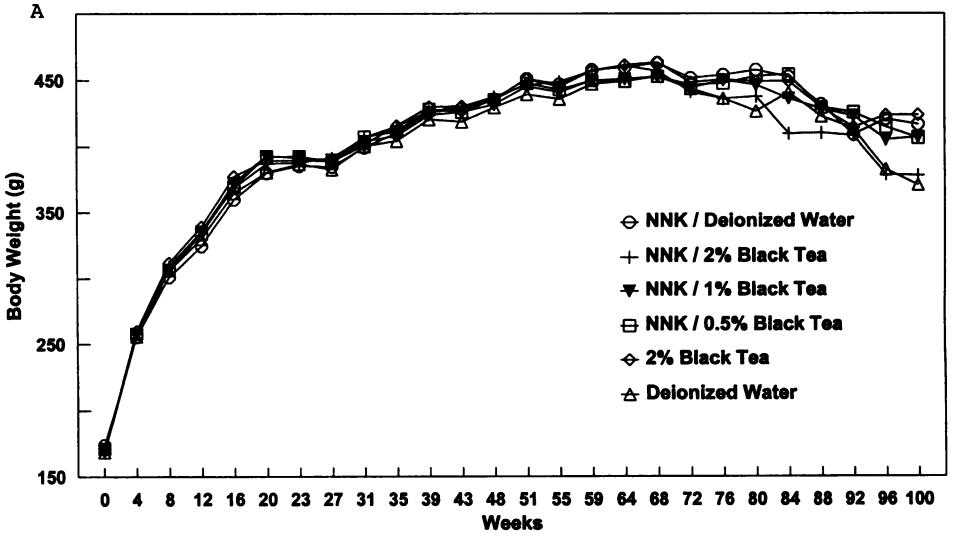
At termination, 70% of the animals in group 5 were still alive as compared to 20–40% in other groups. Although a small number of rats were used in this group, these results suggest that caffeine may have a beneficial effect on the life span of rats. The sharp decline of survival in group 2a from week 73 to 89 raised the question whether the inhibition of lung tumorigenesis observed in this group was due to early death in this group. Of the 17 animals that died prior to week 89, 5 had lung tumors. Of the nine rats that died after week 89, none had developed lung tumors. Therefore, the incidence of lung tumors in group 2a was not decreased as a result of the low survival rate because all of the lung tumors had developed prior to week 89.

Although the lung is the major target organ, NKK also induces tumors in the liver and nasal cavity. The incidences of liver tumors of all groups are presented in Table 3. In group 1, 11 of 32 animals developed hepatic tumors (34%). All of the tumor-bearing animals

Table 1 *Animal survival chart*

Group no.	Treatment	Week[a] 0	51	73	89	96	101
1	NNK	32[b]	32 (100)[c]	31 (97)	23 (72)	13 (41)	7 (22)
2a	NNK/2.0% black tea	26	25 (96)	21 (81)	9 (35)	9 (35)	6 (23)
2b	NNK/1.0% black tea	26	26 (100)	26 (100)	14 (54)	13 (50)	8 (31)
2c	NNK/0.5% black tea	26	26 (100)	22 (85)	14 (54)	10 (38)	8 (31)
3a	NNK/caffeine (680 ppm)	20	20 (100)	19 (95)	14 (70)	7 (35)	5 (25)
3b	NNK/caffeine (170 ppm)	20	18 (90)	17 (85)	9 (45)	7 (35)	4 (20)
4	2.0% black tea	10	10 (100)	9 (90)	8 (80)	5 (50)	3 (30)
5	Caffeine (680 ppm)	10	10 (100)	10 (100)	8 (80)	8 (80)	7 (70)
6	Control	10	10 (100)	9 (90)	7 (70)	4 (40)	4 (40)

[a] Representative weeks during the bioassay when mortality first noted, at week 51 and at other appropriate intervals.
[b] Number of animals remaining in the group.
[c] Numbers in parentheses represent percentage survival.



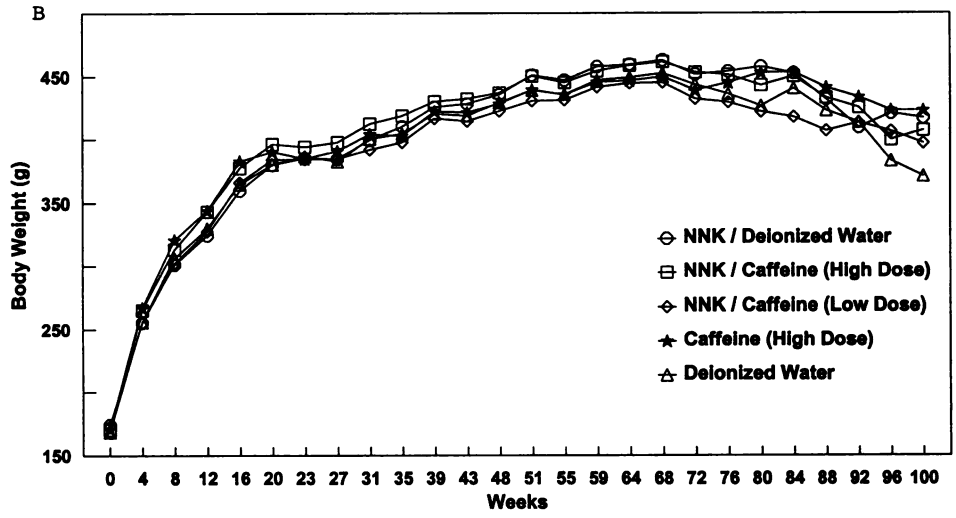


Fig. 1. Growth curves of all groups during the entire period of bioassay. *A*, black tea-treated groups. *B*, caffeine-treated groups.

had adenomas, and only two developed hepatocellular carcinomas. The only group that showed a significant decrease in tumor incidence was group 2a, with a 12% incidence ($P < 0.05$). A total of 11 animals developed liver adenomas in group 1, whereas there were only two in group 2a ($P < 0.05$). Again, the lower concentrations of black tea did not affect the liver tumor formation in the NNK-treated groups. In the nasal mucosa (Table 4), 19% of the animals in group 1 developed tumors, all of which were benign. *Aspergillus* infestation in the nasal cavity did not seem to influence the tumor incidence. The incidences of nasal cavity tumors were not significantly different across all treatment groups.

Discussion

This study is the first to demonstrate the inhibition of lung tumorigenesis by black tea and caffeine in F344 rats during a lifetime 2-year bioassay. The results show that 2% black tea not only significantly reduced the incidence of lung cancer but also decreased the incidence of liver cancer in NNK-treated rats. Previous studies have attributed the inhibitory effect of black tea mainly to its polyphenolic compounds, such as theaflavins, thearubigins, and catechins, because decaffeinated black tea or its theaflavins is also capable of inhibiting lung tumorigenesis in NNK-treated A/J mice (5, 18). The most interesting and perhaps the most important finding of this study is the remarkable inhibitory effect of caffeine against lung tumorigenesis when it is given in drinking water at a concentration similar to that found in the 2% black tea. These results reaffirm our earlier report that caffeine decreased the lung tumor multiplicity in NNK-treated A/J mice slightly but significantly (3). The extent of inhibition observed in that study was much less than that seen here, although the concentration of caffeine (1150 ppm) was nearly 2-fold of that used in this study. Furthermore, two dose levels of caffeine were tested in the present bioassay, whereas only one dose level was tested in the mouse study. Another major difference between these two bioassays is that the growth rate was significantly retarded in the caffeine-treated mice; however, no such effect was seen in rats. These results suggest that caffeine appears to be important for tumor inhibition by black tea. The lack of inhibition in groups 2b and 2c (1 and 0.5% tea) as compared to group 2a (2% tea) indicates a dose-dependent effect. A similar dose effect was observed in the caffeine groups 3a and 3b; the inhibition in group 3b, although not statistically significant, was less than that in

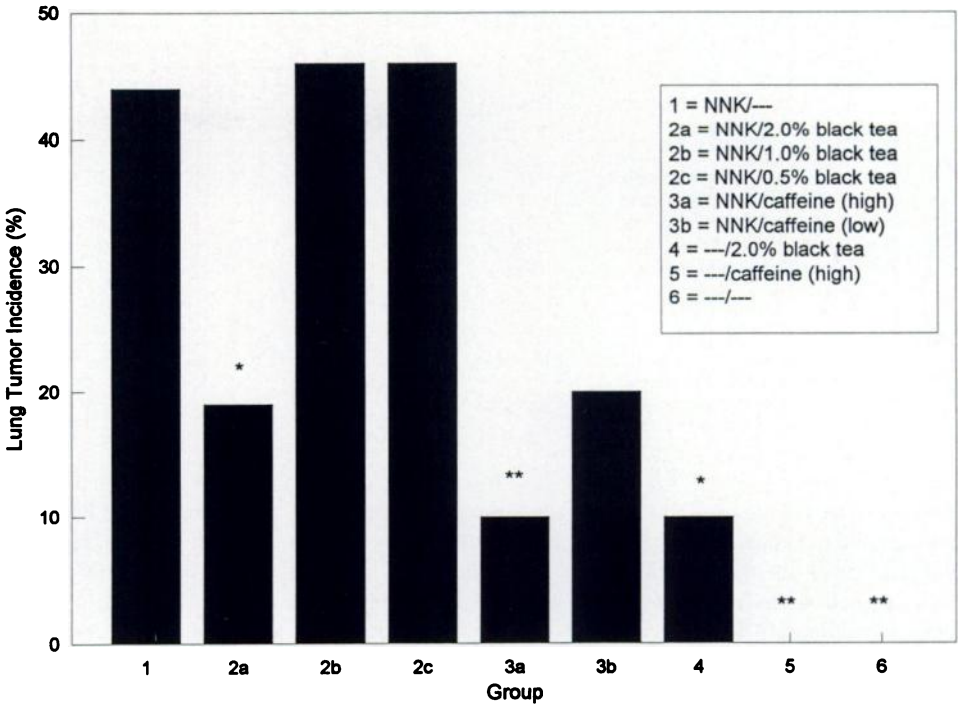


Fig. 2. Lung tumor incidence of the control groups and groups given black tea or caffeine in drinking water. Lung tumors include adenomas, adenocarcinomas, and adenosquamous carcinomas. *, $P < 0.05$; **, $P < 0.01$ as compared to the NNK group (group 1).

group 3a. This study showed that, in addition to the polyphenolic compounds in black tea, caffeine seems to contribute significantly to its inhibitory activity against lung carcinogenesis in F344 rats. Because consumption of caffeine is widespread, these results underscore the importance to investigate its potential protective role against lung cancer caused by cigarette smoking.

The bioassay described in this study was designed to mimic, to some extent, the human situation, in that black tea was given as

Table 2 *Incidence of lung tumors after treatment with NNK and/or test compounds*

Group no.	Treatment	Total no. of rats	No. of rats with tumors[a]			Total no. of rats with tumors	Tumor incidence (%)
			Adenoma	Adenocarcinoma	Adenosquamous carcinoma		
1	NNK	32	10	7	1	15	47
2a	NNK/2.0% black tea	26[b]	4	1	0	5	19
2b	NNK/1.0% black tea	26[c]	9	2	1	11	42
2c	NNK/0.5% black tea	26	7	5	1	13	50
3a	NNK/caffeine (high)	20	1	1	0	2	10
3b	NNK/caffeine (low)	20[c]	3	1	0	4	20
4	2.0% black tea	10	1	0	0	1	10
5	Caffeine (high)	10	0	0	0	0	0
6	Control	10	0	0	0	0	0

[a] Significant differences were found in the following group comparisons: for total tumor incidence, group 1 *vs.* 2a or 4 ($P < 0.05$) and group 1 *vs.* 3a, 5, or 6 ($P < 0.01$); for adenoma, group 1 *vs.* 3a, 5, or 6 ($P < 0.05$); and for adenocarcinoma, group 1 *vs.* 2a ($P < 0.05$).
[b] Two animals were autolyzed (these two animals did not have tumors).
[c] One animal was autolyzed (this animal did not have tumors).

Table 3 *Incidence of liver tumors after treatment with NNK and/or test compounds*

Group no.	Treatment	Total no. of rats	No. of rats with tumors[a]		Total no. of rats with tumors	Tumor incidence (%)
			Adenoma	Hepatocellular carcinoma		
1	NNK	32[b]	11	2	11	34
2a	NNK/2.0% black tea	26[c]	2	1	3	12
2b	NNK/1.0% black tea	26[b]	7	2	8	31
2c	NNK/0.5% black tea	26[b]	6	0	6	23
3a	NNK/caffeine (high)	20	7	1	8	40
3b	NNK/caffeine (low)	20[b]	4	1	5	25
4	2.0% black tea	10	0	0	0	0
5	Caffeine (high)	10	1	1	1	10
6	Control	10	1	1	1	10

[a] Significant differences were found in the following group comparisons by χ^2 test for equality of proportions: for total tumor incidence, group 1 *vs.* 2a or 4 ($P < 0.05$); for adenoma, group 1 *vs.* 2a ($P < 0.05$). No significant differences were found in the incidence of hepatocellular carcinoma.
[b] One animal was autolyzed (this animal did not have tumors).
[c] Two animals were autolyzed (these animals did not have tumors).

Table 4 *Incidence of nasal cavity tumors after treatment with NNK and/or test compounds*

Group no.	Treatment	Total no. of rats	No. of rats with tumors				Total no. of rats with tumors[c]	Tumor incidence (%)
			Aspergillus (+)		*Aspergillus* (−)			
			Benign[a]	Malignant[b]	Benign[a]	Malignant[b]		
1	NNK	32	2	0	4	0	6	19
2a	NNK/2.0% black tea	26	2	1	2	1	6	23
2b	NNK/1.0% black tea	26	0	1	3	1	5	19
2c	NNK/0.5% black tea	24[d]	1	1	4	1	6	25
3a	NNK/caffeine (high)	20	3	0	2	0	5	25
3b	NNK/caffeine (low)	20	0	0	2	1	3	15
4	2.0% black tea	10	0	0	0	0	0	0
5	Caffeine (high)	10	0	0	1	0	1	10
6	Control	10	1	0	0	0	1	10

[a] Includes "hyperplasia" (polypoid, papillary, sessile, nodular, and stem cell), adenoma, papilloma, and polyp.
[b] Includes carcinoma and adenocarcinoma.
[c] There were no significant differences among the groups.
[d] Nasal cavity tissue not available for two animals.

drinking water for the period of carcinogen exposure. This protocol, however, did not allow us to distinguish whether the inhibitory effect of black tea is on the initiation phase or postinitiation stage of NNK-induced carcinogenesis. Decaffeinated black tea inhibited lung tumor formation in A/J mice treated with NNK, but it did not significantly alter the levels of DNA methylation by NNK in the lung DNA (5). These results suggest that the inhibition by decaffeinated black tea most likely involves the action by its polyphenolic compounds during the postinitiation stages of lung carcinogenesis. The role of antioxidant polyphenols in black tea in lung tumor inhibition may be examined by administering decaffeinated black tea. The induction of hepatic cytochrome P450 enzymes, such as 1A2, 1A1, and 2B1, has been described in rats given either green tea or black tea (19–21). Caffeine has been identified as the active compound in tea responsible for enzyme induction in the liver (21). Although the mechanism of the inhibition of lung tumorigenesis by caffeine is not known, it is postulated that the induction of cytochrome P450 enzymes in the rat liver may, in part, account for the inhibition because the increased metabolism of NNK in the liver will result in its decreased bioavailability to the lung. This mechanism has been shown to underlie the inhibition of NNK-induced lung tumorigenesis by indole-3-carbinol, a potent inducer of hepatic cytochrome P450 enzymes (22). It is possible, however, that other mechanisms may also be involved because caffeine is known to have a broad range of biochemical and physiological activities (23). It is well documented that caffeine modulates carcinogenesis at various organ sites, including liver, skin, lung, and mammary gland, in animals treated with carcinogens (23). Treatment with caffeine significantly decreased lung tumor formation in mice treated with the precursors of *N*-nitrosomorpholine and with *N*-nitrosodiethylamine, 4-nitroquinoline-1-oxide, and urethane (24–27). Caffeine seems to invariably protect against lung tumorigenesis in both mice and rats treated with carcinogens. In contrast, caffeine can either stimulate or inhibit carcinogen-induced mammary gland tumorigenesis, depending on the species and strains and the phases during which it is administered (28). In recent years, caffeine has been shown to inhibit gastric tumor promotion by NaCl in rats (29), despite enhancing the pancreatic tumorigenesis caused by *N*-nitrosobis(2-oxopropyl)amine in hamsters when administered during the postinitiation phase (30). Caffeine also protected against UV light-induced skin tumorigenesis and seemed to be actively involved in the protection of UV-induced skin tumorigenesis by green and black tea (31, 32)

Despite numerous studies in rodents, epidemiological data on the effect of consumption of black tea and caffeine on human lung cancer are scanty and inconclusive. Most of the available information indicates that there is no apparent relationship between black tea consumption and lung cancer risk (9). A more recent study showed that consumption of black tea was inversely associated with lung cancer among smokers. However, this association is diminished after smoking and intake of fruits and vegetables are taken into account (10). Similarly, epidemiological investigations have yet to clarify the relationship of caffeine intake (*e.g.*, coffee consumption) on lung cancer. In this regard, the protective effect of black tea and caffeine against lung cancer demonstrated in this study presents a strong basis to warrant large-scale, well-designed epidemiological studies to further assess the role of black tea and caffeine in lung cancer among smokers.

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