Black tea constituents, theaflavins, inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK)-induced lung tumorigenesis in A/J mice

Guang-yu Yang, Zhijian Liu, Darren N.Seril, Jie Liao, Wei Ding, Sungbin Kim, Flordeilia Bondoc and Chung S.Yang1

Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, NJ 08855-0789, USA

1To whom correspondence should be addressed

The present study investigated the inhibitory activity against lung tumorigenesis by a group of characteristic black tea polyphenols, theaflavins. In a short-term study, female A/J mice were treated with a single dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK; 103 mg/kg b.w., i.p.) on day 0, and 0.1 and 0.3% theaflavins were administered as the sole source of drinking fluid starting 24 h after NNK treatment. The proliferation index of the lung tissues was measured by the incorporation of bromodeoxyuridine (BrdU) immunohistochemically. The highest NNK-induced proliferation rate of bronchiolar cells, observed on day 5, was significantly decreased by 0.3% theaflavins (proliferation index, 1.51 ± 0.08 versus 2.35 ± 0.16). In a long-term lung tumorigenesis study, pulmonary adenomas were observed in 100% (30/30) of the mice at week 16 after NNK treatment. Administration of theaflavins (0.1%) as the sole source of drinking fluid, starting 2 days after the NNK treatment until the termination of the experiment, significantly reduced the tumor multiplicity and volume by 23% (8.5 ± 0.6 versus 6.5 ± 0.6 tumors/mouse) and 34% (0.08 versus 0.05 mm³ per tumor), respectively. The proliferation index in lung adenomas was also significantly inhibited by theaflavins. The present work demonstrates the inhibitory action of theaflavins against NNK-induced pulmonary hyperproliferation and tumorigenesis.

Introduction

Lung cancer is the leading cause of cancer death in the United States and is also one of the most common cancers worldwide (1). Tobacco smoking is the most important risk factor for lung cancer (2–4). Although tobacco smoking cessation is an important public health goal, its success is limited to certain populations. Even if it is successful, ex-smokers are still at risk for developing lung cancer and the incidence of lung cancer will still remain very high in the coming decades (5). Therefore, additional lung cancer prevention strategies, such as chemoprevention, are also important. Chemoprevention is defined as the administration of natural or synthetic compounds to inhibit, block or reverse the process of carcinogenesis (6,7).

Tea (Camellia sinensis) is a promising agent for the chemoprevention of cancer (8–10). Although epidemiological studies concerning the relationship between tea consumption and cancer risk have been inconclusive (8,10–13), inhibition of carcinogenesis by tea has been demonstrated in many animal models, including those involving cancers of the lung, skin, esophagus and stomach (8–10,14). In the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK*)-induced lung tumorigenesis model in A/J mice, administration of green tea, black tea, or the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) significantly inhibits tumorigenesis (15–17).

Tea is one of the most popular beverages worldwide. The most commonly consumed tea in the United States and Western countries is black tea. Theaflavins are the characteristic polyphenol constituents in black tea. These polyphenols are formed from the oxidation of the tea flavanols (catechins and gallatecachtsins) by polyphenol oxidase during the manufacturing of black tea in a process known as ‘fermentation’ (18). The fermentation process causes the reduction, by several fold, in the quantities of some of the characteristic green tea flavanols, such as (-)-epigallocatechin gallate, (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epicatechin. Usually, theaflavins account for 1.5% of the water extractable materials from black tea. Ten to twenty per cent of the water extractable materials is known as thearubigens, which are poorly characterized. Theaflavins are responsible for the characteristic reddish color and astrignency of black tea (18,19). The astrignency is due to the precipitation of the mucous glycoproteins in the mouth by polyphenols (20). The theaflavins are a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin-3,3'-digalate, with structures shown in Figure 1 (18). Theaflavins and green tea flavanols are known to have antioxidative activities due to radical scavenging and metal chelating functions (20–23). This activity is believed to be one of the mechanisms of anti-mutagenesis and anti-carcinogenesis (24,25).

The anti-oxidative and anti-mutagenic activities of theaflavins have been demonstrated recently (21,23). In the present study, we investigated the effect of theaflavins on NNK-induced early pulmonary proliferation and lung tumorigenesis in A/J mice. In order to determine whether the treatment with theaflavins influences the nutritional status of vitamins A and E, which may subsequently affect carcinogenesis, the plasma levels of retinol and α-tocopherol were measured.

Materials and Methods

Materials

NNK was obtained from Chemsyn Science Laboratories (Lenexa, KS). The theaflavins preparation was kindly provided by the Thomas J.Lipton Company (Englewood Cliffs, NJ). This preparation was a mixture of theaflavin (21%), theaflavin-3-gallate (30%), theaflavin-3'-gallate (15%), and theaflavin-3,3'-digallate (28%). The ALN-76 diet was purchased from Research Diets, Inc. (New Brunswick, NJ). Bromodeoxyuridine was purchased from Zymed Laboratory, Inc. (San Francisco, CA).

Treatment of animals

Female A/J mice (6 weeks old, from Jackson Laboratory, Bar Harbor, ME) were acclimatized in our laboratory for 2 weeks and then treated with a single dose of NNK (103 mg/kg, b.w., i.p.) or saline. The animals were fed an ALN-76 diet and given water ad libitum. They were maintained in air conditioned...
per time point. Bromodeoxyuridine (BrdU) was dissolved in sterile normal saline until the termination of the experiment (days 5 and 7). De-ionized water was given to the mice as the sole source of drinking fluid 24 h after NNK treatment and an alternating 12-h light/dark cycle. De-ionized water was administered to the control mice. The experiment was performed with 5 mice per time point. Bromodeoxyuridine (BrdU) was dissolved in sterile normal saline and administered i.p. at 50 μg/g body wt to the mice 2 h before death. The lungs were removed, inflated, fixed in Carnoy’s fixative and embedded with paraffin.

The second experiment was designed to study the inhibition of NNK-induced lung tumorigenesis by theaflavins. Solutions of theaflavins (0.1% and 0.3% dissolved in de-ionized water) were made fresh and transferred to 80% ethanol 3 and 24 h after Carnoy’s and formalin fixation, respectively. Visible tumors on the surface of the lungs were counted. The BrdU-injected mouse lungs were fixed with Carnoy’s solution and the lungs were fixed with 10% buffered formalin. The lungs were embedded in paraffin blocks.

The first experiment was designed to study the inhibitory effect of theaflavins on NNK-induced early pulmonary proliferation in A/J mice. Solutions of theaflavins (0.1 and 0.3% dissolved in de-ionized water) were made fresh and given to the mice as the sole source of drinking fluid 24 h after NNK treatment until the termination of the experiment (days 5 and 7). De-ionized water was administered to the control mice. The experiment was performed with 5 mice per time point. Bromodeoxyuridine (BrdU) was dissolved in sterile normal saline and administered i.p. at 50 μg/g body wt to the mice 2 h before death. The lungs were removed, inflated, fixed in Carnoy’s fixative and embedded with paraffin.

Histopathological analysis

For the NNK-induced lung tumorigenesis experiment, serial sections (5 μm) were made for each tissue block (total 30 sections/block). The tissue slides from the serial sections (section numbers 1, 15 and 30 for each lung) were routinely stained with hematoxylin and eosin for histopathological analysis. Pulmonary lesions were categorized as hyperplasia and adenoma based on established criteria [26,27].

Immunohistochemistry for BrdU-incorporated proliferative cells

The BrdU-incorporated proliferative cells were determined immunohistochemically. Three slides from the serial sections of each block (numbering blocks 2, 16 and 29) were immunostained using a rat monoclonal anti-BrdU antibody (Harlan Bioproducts, Indianapolis, IN) and the avidin-biotin peroxidase complex method (Elite ABC/rat IgG kit, Vector Laboratories, Burlingame, CA). The dewaxed tissue slides were denatured with 0.5 M HCl for 15 min. After quenching endogenous peroxidase with 3% H2O2 and minimizing nonspecific binding by incubation with 1% normal rabbit serum, the tissue slides were sequentially incubated at room temperature with primary antibody (5 μg/ml rat anti-BrdU antibody) for 1 h, biotinylated secondary antibody (rabbit anti-rat antibody, 1:200 dilution) for 30 min and avidin-biotin peroxidase complex (1:100 dilution) for 45 min. Diaminobenzidine (Sigma Co., St Louis, MO) was used as a chromagen. The slides were washed with phosphate-buffered saline between incubations and counterstained by hematoxylin.

Negative controls were conducted by replacing the primary antibody with phosphate-buffered saline and normal rat serum. A portion of the small intestine from the same mouse was used as a positive-control tissue on every slide. The proliferation index, defined as the percentage of BrdU-positive cells in the bronchiolar epithelium, was determined for each slide using a Nikon research microscope combined with an Image-Pro Plus system (Media Cybernetics, Silver Spring, MD). Under a ×20 objective lens, at least 10 bronchiolar and 10 alveolar regions of a slide, and more than three fields for each lesion (adenoma or hyperplasia), were counted. The total number of cells counted was >2000.

Plasma retinol and α-tocopherol

The concentrations of retinol and α-tocopherol were determined by high performance liquid chromatography (HPLC; 28,29) using a Supelco LC 18 column (4.6 × 15 mm, 5 μ, 100 Å) and a Waters 490E multiwavelength detector. Elution was carried out using a modified solvent system of 50% absolute ethanol and 50% acetonitrile at a flow rate of 0.9 ml/min. Retinyl butyrate was used as an internal standard. The elution pattern was monitored by measuring the absorbance at 300 nm on channel 1 for retinyl-α-tocopherol, at 325 nm on channel 2 for retinol, and at 450 nm on channel 3 for lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene.

Statistical analysis

The data on the proliferation index, tumor multiplicity and volume, and concentrations of fat soluble vitamins were analysed in comparison to the control group by the Student’s t-test using computer software (SigmaStat Version 1.01).

Results

Inhibition of NNK-induced early bronchiolar hyperproliferation by theaflavins

NNK-induced early bronchiolar hyperproliferation in A/J mice has been studied previously in our laboratory and the highest proliferation rates in the bronchiolar epithelia were observed on day 5 after NNK treatment (30). In the present study, solutions of theaflavins (0.1 and 0.3%) were administered to the mice as the drinking fluid starting 24 h after NNK treatment. On days 5 and 7, the proliferation index of the bronchiolar epithelial cells was measured by BrdU-incorporation immunohistochemically (Figure 2a,b). Consistent with previous results, hyperproliferation was observed in bronchiolar epithelial cells, but not in alveolar epithelial cells. NNK-induced bronchiolar epithelial cell proliferation was significantly inhibited on both days 5 and 7 by treatment with 0.3% theaflavins (Table I). The proliferation indices were also decreased by 0.1% theaflavins, but the results were not statistically significant.

Inhibition of NNK-induced lung tumorigenesis by theaflavins

In the NNK-induced lung tumorigenesis experiment, the mice received 0.1% theaflavins in the drinking water as the sole source of drinking fluid, starting 2 days after a single dose of NNK treatment and continuing until the termination of the experiment. At week 16, lung tumors were found in all of the NNK-treated mice in both the positive control group and the theaflavins-treated group, but lung tumors were not observed in the mice in either the water- or theaflavins-negative control group. Significant inhibition of tumor multiplicity and tumor volume (by 24 and 33%, respectively) was observed in the mice treated with theaflavins (Table II). The gross lung tumors were further confirmed by histopathological analysis. The tumors were identified as monomorphic and well-differentiated alveolar adenomas without nuclear atypia (Figure 3a). Compression of the adjacent pulmonary parenchyma by the adenoma was frequently observed. Occasionally, infiltrative lymphocytes surrounding the adenoma were found. The morphology of the adenomas observed in the mice treated with NNK-plus-theaflavins was similar to that in mice treated with NNK. Alveolar hyperplasia was observed in a few of the mice in both groups of animals (Figure 3b). Hyperplasia occurred in the alveolar regions as single or plural layers of proliferative epithelial cells along intact alveolar septae with irregular and...
Inhibition of NNK-induced lung tumor by theaflavins

Fig. 2. BrdU-immunohistochemistry for NNK-induced early pulmonary hyperproliferation. (a) Lung from a negative control mouse on day 5, a BrdU-labeled cell was located in the bronchiolar epithelium (microscope setting ×400). (b) Lung from an NNK-treated mouse on day 5, several BrdU-labeled cells were detected in the bronchiolar epithelium (×250).

Fig. 3. Typical NNK-induced pulmonary lesions in A/J mice. (a) An adenoma (×250) and (b) hyperplasia (×400) in mice from the NNK-treated group at week 16.

Table I. Inhibitory action of theaflavins against NNK-induced early bronchiolar epithelial cell proliferation. Mice were treated as described in Materials and methods, and were killed on days 5 and 7. The values are the mean ± SE of the proliferation index in five mice for each group (with percentage inhibition shown in parentheses)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proliferation index</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 7</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.16%</td>
<td>0.18%</td>
<td></td>
</tr>
<tr>
<td>NNK</td>
<td>2.35% ± 0.16%</td>
<td>1.70% ± 0.07%</td>
<td></td>
</tr>
<tr>
<td>NNK + Theaflavins (0.1%)</td>
<td>1.63% ± 0.13% (31%)</td>
<td>1.46% ± 0.05%</td>
<td></td>
</tr>
<tr>
<td>NNK + Theaflavins (0.3%)</td>
<td>1.51% ± 0.08%* (36%)</td>
<td>1.31% ± 0.06%* (23%)</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from NNK group by Student’s t-test (P<0.05).

Table II. Inhibition of NNK-induced lung tumors in A/J mice by theaflavins. Solutions of theaflavins (0.1%) were administered to the mice as the sole source of drinking fluid 2 days after a single dose of NNK (100 mg/kg b.w., i.p.) until the end of the experiment. Tumor multiplicity and volume (per tumor) are the mean ± SE of 10 and 30 mice in the negative control and NNK-treated groups, respectively (with percentage inhibition by theaflavins shown in parentheses)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tumor incidence (%)</th>
<th>Tumor Multiplicity</th>
<th>Tumor Volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-control</td>
<td>0 (0/10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Theaflavins-control</td>
<td>0 (0/10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NNK</td>
<td>100 (30/30)</td>
<td>8.5 ± 0.6</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>NNK + Theaflavins</td>
<td>100 (30/30)</td>
<td>6.5 ± 0.6* (24%)</td>
<td>0.05 ± 0.01* (38%)</td>
</tr>
</tbody>
</table>

*Significantly different from the NNK group by Student’s t-test (P<0.05).

Inhibition of cell proliferation in lung hyperplasia and adenomas by theaflavins

The proliferative cells with BrdU incorporation were measured by immunohistochemical analysis. In the water- and theaflavins-control mice, ‘scattered’ BrdU-labeled cells were observed in the bronchiolar and alveolar epithelia, and the proliferation index was <0.5%. The proliferation indices in the morphologically normal bronchiolar and alveolar epithelia of the mice treated with NNK and NNK-plus-theaflavins were also at the same level. The number of BrdU-labeling proliferative cells increased in NNK-induced alveolar hyperplasia and adenomas. Figure 4 shows immunostaining-positive BrdU-labeling cells in the adenomas from a mouse treated with NNK (Figure 4a) and from a mouse treated with NNK-plus-theaflavins (Figure 4b). The inhibitory effect of theaflavins on cell proliferation in NNK-induced lung hyperplasia and adenomas is summarized in Table III. The proliferation indices in both the hyperplasia and adenomas were significantly decreased by theaflavins (44 and 31%, respectively).

General nutritional status and health conditions

In the NNK-induced lung tumorigenesis experiment, the body wts of the mice were measured weekly and are shown in Figure 5. No significant difference in the body wts was observed among the different groups. Similar drinking fluid consumption was also observed, with an average of 2.5 ml of...
theaflavins-treated mouse, only a few BrdU-labeled cells were observed. (38x634)

percentage inhibition shown in parentheses)

index in lung hyperplasia and adenomas in 15 mice for each group (with

BrdU-labeled cells were observed. (38x52)

were collected from decapitated mice at 16 weeks. Values are the mean

mg/kg b.w., i.p.) until the end of the experiment. The plasma samples were

as the sole source of drinking fluid 2 days after a single dose of NNK (100

NNK and theaflavins. Theaflavins preparation was administered to the mice

were killed at 16 weeks. The values are the mean

plasma levels

of

α

1

Vitamins Water-control Theaflavins NNK NNK + Theaflavins

were collected at the time of sacrifice, and the plasma levels

drinking fluid consumed per day per mouse. Blood samples

were collected at the time of sacrifice, and the plasma levels of α-tocopherol and retinol were measured using the HPLC method, and the data are shown in Table IV. No significant difference in the plasma levels of α-tocopherol and retinol was observed. Carotenoids and γ-tocopherol were not detected

in the samples, apparently due to the lack of these compounds in the AIN-76 diet.

Discussion

The present study demonstrated that administration of the black tea polyphenol component, theaflavins, decreased both lung tumor multiplicity and volume in A/J mice (Table II). This inhibitory action of theaflavins may be enacted, at least in part, by inhibiting cell hyperproliferation in the early and late stages of carcinogenesis. NNK is known to be activated to alkylating agents which attack DNA and cell division is needed to fix mutations caused by modified DNA in daughter cells. With the suppression of NNK-induced early bronchiolar hyperproliferation by theaflavins, the mutation events may be reduced. Because the activation of NNK is known to be mostly completed within 24 h after administration, treatment with theaflavins 24 or 48 h after the NNK dose is not expected to significantly affect the carcinogen activation process. It is interesting that the treatment with theaflavins also inhibited the proliferation index in lung hyperplasia and adenomas. This anti-proliferative activity was also observed in our previous studies with black tea and black tea polyphenols (30). To our knowledge, this is the first demonstration of the anti-proliferative and anti-tumorigenic activities of theaflavins in the lung. The relative contribution by each of the components in the mixture of theaflavins to the presently observed activity remains to be determined.

Many studies have demonstrated that green tea and green tea preparations inhibit chemically-induced tumors in different animal models (8–10,14). A few studies have also shown that a water extract of black tea has similar inhibitory activities (15,16). This inhibitory activity of black tea could be due to theaflavins, tea flavanols or some other constituents, and most likely a combination of some of the constituents. In our previous work, we found that administration of 0.3% decaffeinated black tea (0.3 g of dehydrated water extract of black tea reconstituted in 100 ml of warm water) after a dose of NNK reduced tumor multiplicity by 62% (15). That tea solution contained ~0.003–0.005% theaflavins. Although quantitative information is still lacking, a rough comparison of those previous findings and the present result (0.1% theaflavin reduced lung tumor multiplicity by 23%) suggests that the inhibitory effect is due to the combined effects of theaflavins and other components in black tea.

The mechanisms by which theaflavins exert their inhibitory effect is not known. Recent studies have indicated that the inhibition of AP-1 activity in JB6 cells may be related to the anti-growth activity of theaflavins (31). It is known that in NNK-induced lung tumorigenesis, the Ki-ras gene is mutated, and that this may be a key factor which not only initiates, but also promotes, the development of lung tumors (27,32,33). It has been suggested recently that superoxide production may be related to the Ki-ras-driven oncogenic process, and that anti-oxidants may inhibit this process (34). Whether this proposed mechanism is applicable to our model remains to be investigated.

In the search for new cancer chemopreventive agents that has been conducted over the past several years, only a few agents have been found to act during the promotion stage of carcinogenesis, and tea is one of these agents. The present study demonstrated that administration of 0.1% theaflavins in the drinking water starting 2 days after NNK treatment

Table III. Inhibition of cell proliferation in hyperplasia and adenomas by theaflavins. Mice were treated as described in Materials and methods and were killed at 16 weeks. The values are the mean ± SE of the proliferation index in lung hyperplasia and adenomas in 15 mice for each group (with percentage inhibition shown in parentheses)

Table IV. The levels of plasma fat soluble vitamins in the mice treated with NNK and theaflavins. Theaflavins preparation was administered to the mice as the sole source of drinking fluid 2 days after a single dose of NNK (100 mg/kg b.w., i.p.) until the end of the experiment. The plasma samples were collected from decapitated mice at 16 weeks. Values are the mean ± SD of six plasma samples (each sample was pooled from five mice) in the NNK and NNK-plus-theaflavins groups, and three samples (each samples was pooled from three mice) of the water- and theaflavins-control groups

![Fig. 4. BrdU-immunohistochemistry for lung adenomas (counterstained with hematoxylin, ×400). (a) An adenoma from an NNK-treated mouse, several BrdU-labeled cells were observed. (b) An adenoma from an NNK-plus-theaflavins-treated mouse, only a few BrdU-labeled cells were observed.](Image 45x467 to 283x621)

![Fig. 5. Effect of long-term theaflavins administration on body wt. The body wt was determined weekly. No difference in the body wt between the different groups was observed.](Image 47x675 to 281x759)
significantly inhibited NNK-induced lung tumorigenesis, as determined by both tumor multiplicity and volume. The body wt and plasma nutrients were not affected by 0.1% theaflavins. This anti-carcinogenic action, together with the anti-proliferation activity, suggest the potential usefulness of theaflavins in the chemoprevention of human cancer, and this possibility should be investigated in human lung cancer prevention trials.

Acknowledgements
This study was supported by NIH grant CA56673 and NIEHS Center Grant ES05022. The authors would like to thank the Thomas L.J. Lipton Company for providing the theaflavins, Dr Janelle Landau for helpful discussion, and Ms Dorothy Wong for excellent secretarial assistance in the preparation of this manuscript.

References

Received on May 5, 1997; revised on August 4, 1997; accepted on August 14, 1997