ABSTRACT

The carcinogenic potential of caffeic acid was investigated in both sexes of F344 rats and C57BL/6N × C3H/HeN F₁ mice. After groups of 30 animals received diet containing 0 and 2.0% caffeic acid for 104 weeks in rats or 96 weeks in mice, detailed histopathological examination revealed induction of forestomach squamous cell papillomas or carcinomas in rats at high incidence (77% for males; 80% for females) and in mice at low incidence (13% for males; 3% for females). Invasion to the abdominal cavity of these squamous cell carcinomas was observed in three rats and two mice. In addition, renal tubular cell hyperplasias and adenomas, clearly related to toxic lesions, were found in treated rats at high incidence for males (73 and 13%) and low incidence for females (20 and 0%). In mice, renal tubular cell hyperplasias and tumors also occurred in treated females (97 and 28%), and at a lower incidence in treated males (27 and 3%). No toxic renal injuries were apparent in mice. Alveolar type II cell tumors also developed in treated male mice (27%) with statistical significance. Thus, the current investigation showed caffeic acid to exert carcinogenic activity for the forestomach squamous cell epithelium in both sexes of F344 rats and C57BL/6N × C3H/HeN F₁ mice, for the renal tubular cell in male rats and female mice, and for the alveolar type II cell in male mice.

INTRODUCTION

Caffeic acid (3,4-dihydroxycinnamic acid), a natural phenolic antioxidant, is widely distributed in vegetables, fruits, and beverages, and is therefore an integral part of the human diet (1–3). Naturally occurring phenolics, including caffeic acid, have attracted interest due to their inhibitory effects on mutagenesis and carcinogenesis induced by strong mutagens and/or carcinogens, as well as on the formation of nitrosamines due to their nitrite trapping activity (1, 4–9). In addition, it has been reported that caffeic acid acts as an antimutagen promoter on forestomach, liver, and skin carcinogenesis in rodents (10–12).

However, our recent preliminary studies revealed caffeic acid to induce marked squamous cell hyperplasia of the forestomach in rats and hamsters after short-term exposure (13, 14). Similar hyperplastic changes of the forestomach were also observed in rats, mice, and hamsters given the antioxidant BHA, an established forestomach carcinogen, in the short term (13–15). Thus tumorigenic activity of caffeic acid on rodent forestomach epithelium was strongly suggested. The aim of the present investigation was to clarify the carcinogenicity of this antioxidant in F344 rats and C57BL/6N × C3H/HeN F₁, hereafter called B6C3F₁ mice, and also to evaluate its chronic toxic potential.

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1 To whom requests for reprints should be addressed.

2 The abbreviation used is BHA, butylated hydroxyanisole.

MATERIALS AND METHODS

Chemicals. The caffeic acid used in this study was purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan. A purity of at least 98% was guaranteed by the manufacturer. The compound was incorporated into commercial powdered diet MF (Oriental Yeast Co., Tokyo, Japan) at concentrations of 0 and 2.0%; analysis of samples showed the actual level of caffeic acid in the food, nominally containing 2.0%, to be 1.76% (analyzed by Japan Food Research Laboratories, Tokyo, Japan).

Animals and Maintenance. One hundred and twenty F344/DuCrj rats (60 males and 60 females) and 120 B6C3F₁, mice (60 males and 60 females) were obtained from Charles River Japan, Inc., Kanagawa, Japan. The animals were 6 weeks old at the commencement of the experiment and were housed five to a plastic cage with hardwood chips for bedding. The room temperature was maintained at 22 ± 2°C and the relative humidity at 60 ± 10% with a 12-h light, 12-h dark cycle. A positive air pressure was maintained with more than 15 air changes/h.

Long-Term Feeding Studies in Rats and Mice. Groups of 30 male and 30 female animals were given diet containing 0 and 2.0% caffeic acid for 104 weeks for rats or 96 weeks for mice. The selection of the dose was based on the results documented in our previous report (13–15). Observations were performed daily for symptoms and mortalities; animals found dead or killed in moribund condition were autopsied. Individual body weights were recorded weekly for the first 14 weeks and then every other week. Food and water consumption levels were measured over the 2-day period before each weighing. At the termination of the studies, surviving animals were deprived of food, but not water, overnight and then were killed under ether anesthesia. For the mice, blood samples were taken from 12 males and 12 females of each group, and routine hematology and blood biochemistry assessments were performed.

Gross observation was performed at necropsy, and detailed examination of the luminal surfaces of stomach and urinary bladder was also carried out by using a dissecting microscope after fixation. The livers and kidneys of each animal were weighed and organ/body weight ratios were determined. A complete histopathology examination was routinely performed on all animals used in both studies.

Statistical Analysis. All measurement data were expressed as mean ± SD and were analyzed where appropriate by the F and t tests or the Welch method. The significance of differences in the incidences of nonneoplastic and neoplastic lesions between the different groups was evaluated by Fischer's exact probability test. All references to statistical significance in this experiment represent 2-tailed P values. Analyses of differences in survival periods between the treated and control groups were performed by using generalized Wilcoxon (16) and Cox-Mantel (17) tests.

RESULTS

Antemortem Examination. No treatment-related clinical signs were apparent in any of the animals during the course of studies. Although not statistically significant, mortality tended to be increased in treated male rats from week 102, and in treated female mice from week 94 to the termination (Fig. 1). Slight retardation of body weight increase was found in treated male and female rats from week 1 onward (Fig. 2). Body weights were significantly reduced from week 58 to the end of study in treated female mice. In contrast, significantly increased body weights were seen in treated male mice from week 86 to the termination (Fig. 2). Food consumption by controls and treated

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animals showed no clear differences. The intake of caffeic acid, calculated from the nominal dietary level, the mean food consumption, and the mean body weight, was higher in the first 3 months than during the remainder of the experiment. Excluding the first 3 months, the average caffeic acid intakes for males and females were 678 and 814 mg/kg/day in rats, and 2120 and 3126 mg/kg/day in mice, respectively. Treated male, but not female, rats drank more water than controls from week 78 to the termination. Remarkable increase in water consumption was observed in treated female mice, but not in males, from week 70 to the end of study. The hematological examination and blood biochemistry analyses did not reveal noteworthy changes in either sex of treated mice (data not shown).

Gross Pathology and Organ Weights. On gross observation, extremely high incidences of forestomach nodules were recorded in both sexes of the treated animals (Fig. 3). Cystic changes of the kidney were found in several treated female mice. Liver:body weight and kidney:body weight ratios were significantly increased in both sexes of treated rats, especially in males. Marked increase of kidney:body weight ratio was also apparent in treated female mice (Table 1).

Stomach Histopathology. Hyperplastic and neoplastic lesions in the forestomach of rats and mice, considered as treatment-related changes, are summarized in Table 2. Squamous cell papillomas and carcinomas (Fig. 4) developed in treated rats (77 and 57% for males; 80 and 50% for females, respectively) and mice (13 and 10% for males; 0 and 3% for females, respectively), but not in control animals. Abdominal cavity invasion by carcinomas (Fig. 5) was observed in 2 male rats and 1 female rat, and in 2 male mice. Although hyperplasia of the forestomach epithelium was found in controls at only low incidence, this lesion developed in almost all treated animals. An adenoma of the glandular stomach was seen in one treated male rat.

Kidney Histopathology. In rats, renal tubular cell hyperplasias were observed in 21 of 30 treated males (70%) and 6 of 30 treated females (20%), and adenomas were found in 4 of 30 treated males (13%) (Fig. 6). These proliferative tubular lesions were associated with an increased incidence and severity of chronic nephropathy. A mesenchymal tumor was noted in 1 treated male. In mice, renal tubular cell hyperplasias (Fig. 7) were observed in 8 of 30 treated males (27%) and 28 of 29 treated females (97%), and adenomas (Figs. 7 and 8) were found in 8 of 29 treated females (28%). One adenocarcinoma was...
Fig. 3. Gross findings for the luminal stomach surfaces in male (A) and female (B) rats treated with 2.0% caffeic acid for 104 weeks. The entire forestomach epithelium is occupied with large masses.

noted in a treated male. No toxic renal lesions were evident in treated mice (Table 3).

Histopathology of Organs Other than Stomach and Kidney. Alveolar type II cell adenomas (7 of 30; 23%) and 1 adenocarcinoma (1 of 30; 3%) were found in treated male mice, the incidence of these lesions being statistically significant when compared to the control value. Other nonneoplastic and neoplastic lesions observed in these studies were similar to those considered usual in aged F344 rats and B6C3F1 mice (data not shown).

DISCUSSION

The natural phenolic antioxidant caffeic acid, while being nongenotoxic (1), was found to possess forestomach carcinogenicity in both sexes of F344 rats and B6C3F1 mice in the present investigation, as predicted from the earlier short-term toxicity test result (13, 14). The carcinogenic activity of caffeic acid for the forestomach epithelium was comparable to that of BHA, an established forestomach carcinogen, in rats and mice. BHA is reported to induce severe squamous cell hyperplasia and increased DNA synthesis in the forestomach epithelium of rats after only short-term exposure (14, 15). In our preliminary oral 4-week study, caffeic acid also caused marked squamous cell hyperplasia in the entire forestomach of F344 rats, whereas other antioxidants such as butylated hydroxytoluene, gallic acid, syringic acid, ferulic acid, and esculin did not (14). It has been clearly demonstrated that proliferative forestomach lesions observed in rats fed 2.0% caffeic acid for 24 weeks are reversible, which was also found to be the case with the other nongenotoxic forestomach carcinogen, BHA (15). However, prolonged administration (60 weeks) of caffeic acid resulted in increased incidences of squamous cell papilloma of the forestomach (15). Furthermore, caffeic acid induced squamous cell hyperplasia, but did not obviously increase DNA synthesis, in forestomach epithelium of Syrian golden hamsters after a 20-week p.o. administration (13). In an initiation-promotion protocol, caffeic acid exerted weak promoting activity on forestom-
Carcinogenicity of Caffeic Acid

Table 1: Final body and relative organ weights of F344 rats and B6C3F1 mice given caffeic acid in the diet (mean ± SD)

<table>
<thead>
<tr>
<th>Species and sex</th>
<th>Diet group</th>
<th>No. of animals</th>
<th>Final body wt (g)</th>
<th>Liver (% of body wt)</th>
<th>Kidneys (% of body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>24</td>
<td>438 ± 38</td>
<td>1.86 ± 1.15</td>
<td>0.47 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>2.0%</td>
<td>18</td>
<td>395 ± 62*</td>
<td>3.02 ± 0.47*</td>
<td>0.89 ± 0.23*</td>
</tr>
<tr>
<td>Rats Male</td>
<td>0%</td>
<td>22</td>
<td>306 ± 42</td>
<td>2.38 ± 0.38</td>
<td>0.62 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>2.0%</td>
<td>21</td>
<td>296 ± 24</td>
<td>2.63 ± 0.26*</td>
<td>0.70 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>22</td>
<td>375 ± 64</td>
<td>5.81 ± 2.57</td>
<td>1.78 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>2.0%</td>
<td>23</td>
<td>41.7 ± 4.5*</td>
<td>4.85 ± 2.11</td>
<td>1.86 ± 0.18</td>
</tr>
<tr>
<td>Mice Male</td>
<td>0%</td>
<td>26</td>
<td>47.2 ± 7.3</td>
<td>3.60 ± 1.02</td>
<td>1.05 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>2.0%</td>
<td>21</td>
<td>39.6 ± 8.0*</td>
<td>4.49 ± 0.73*</td>
<td>1.71 ± 0.38*</td>
</tr>
</tbody>
</table>

* Significantly different from corresponding control value at P < 0.05, 0.01, respectively.

Table 2: Hyperplastic and neoplastic lesions developing in the stomach of F344 rats and B6C3F1 mice fed caffeic acid-containing diet

<table>
<thead>
<tr>
<th>Site and type of lesion</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forestomach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>1 (3)*</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Papilloma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>0</td>
<td>15 (50)*</td>
</tr>
<tr>
<td>Glandular stomach</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenoma</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data are shown as numbers of lesion-bearing animals; incidences as percentages are in parentheses.

ach carcinogenesis in female SD rats initiated with 7,12-dimethylbenz[a]anthracene (18), and strongly promoted that in male F344 rats initiated with N-methyl-N'-nitro-N-nitrosoguanidine. The available evidence thus indicates that development of squamous cell tumors requires continuous feeding (more than 60 weeks) of nongenotoxic forestomach carcinogens, including caffeic acid.

In the present study, renal tubular hyperplasias and adenomas were found in rats, particularly in males, receiving 2.0% caffeic acid. These proliferative lesions usually accompany severe chronic toxicity in the kidney, but are not related to α2-M-globulin nephropathy (19). It has been reported that ethoxyquin (20) and hydroquinone (21), two synthetic antioxidants, also cause renal tubular cell neoplasms in rats after long-term administration. Ethoxyquin also possesses promoting activity on renal carcinogenesis in rats initiated with N-ethyl-N-hydroxyethyl-N-nitrosamine, while the synthetic antioxidants BHA and butylated hydroxytoluene, which are not as toxic for the kidney, did not (15, 22). There are many additional reports concerning modification of renal carcinogenesis by renal toxic agents (23, 24), some of these compounds being shown to induce renal cell tumors in the long term (23). From this evidence, it is strongly suggested that the renal carcinogenicity of caffeic acid in rats might be closely related to its renal toxicity. However, caffeic acid also induced renal tubular cell hyperplasias and tumors in mice, female animals being more susceptible. These proliferative tubular lesions were not accompanied by toxic tubular changes and treated female mice did not show any adverse effects regarding blood biochemical parameters indicative of renal failure, despite high mortality, significant body weight decrease, and markedly increased water ingestion. Renal tubular cell hyperplasias are observed in rats receiving 2.0% caffeic acid in the diet.
CARCINOGENICITY OF CAFFEIC ACID

Fig. 6. Tubular cell adenoma of the kidney in a male rat maintained on diet containing 2.0% caffeic acid. Chronic nephropathy is evident around the adenoma. H&E, original magnification, × 50.

Fig. 7. Tubular cell hyperplasias (small arrow) and an adenoma (large arrow) of the kidney in a female mouse maintained on diet containing 2.0% caffeic acid. H&E, original magnification, × 20.

Fig. 8. Higher magnification of the tubular cell adenoma in Fig. 7. H&E, original magnification, × 50.

Table 3 Nonneoplastic and neoplastic lesions developing in the kidney of F344 rats and B6C3F1 mice fed caffeic acid-containing diet

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Rats</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic nephropathy</td>
<td></td>
<td>20%</td>
<td>30%</td>
</tr>
<tr>
<td>++</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell hyperplasia</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell adenoma</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenchymal tumor</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyelonephritis</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell hyperplasia</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell adenoma</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell adenocarcinoma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Male: 0% 2.0% 0% 2.0%
Female: 30 30 30 30

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Mice</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyelonephritis</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell hyperplasia</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell adenoma</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular cell adenocarcinoma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Male: 28 30 29 29
Female: 29 29 29

* Data are shown as numbers of lesion-bearing animals; incidences as percentages are in parentheses.

Caffeic acid is an integral part of some edible plants, although p.o. intake of this compound in humans is assumed to be at quite low levels (1). While the dose dependence of caffeic acid carcinogenicity was not investigated in the current study, it is strongly suggested that caffeic acid, a natural phenolic antioxidant, would show a high threshold level similar to that found for the synthetic phenolic antioxidant BHA (27, 28).

In conclusion, the present study demonstrated that high dose of caffeic acid can exert forestomach and kidney carcinogenic activity in F344 rats and B6C3F1 mice, and also shows limited lung carcinogenic potential for male mice. However, in terms of risk assessment for humans, the available evidence suggests that the carcinogenic hazard of caffeic acid to humans may be negligible.

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Forestomach and Kidney Carcinogenicity of Caffeic Acid in F344 Rats and C57BL/6N × C3H/HeN F₁ Mice

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