Interactive Effects of Unleaded Gasoline and Estrogen on Liver Tumor Promotion in Female B6C3F1 Mice

Andrew M. Standlee,1 Douglas C. Wolf, and Thomas L. Goldsworthy2

Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina, 27709

ABSTRACT

We tested whether a concentration of unleaded gasoline (UG) vapor that was selectively hepatocarcinogenic in female mice in a chronic bioassay is antiestrogenic and whether liver tumor promotion by UG is secondary to antiestrogenicity. Twelve-week-old female C57BL/6 × C3H F1 mice (hereafter called B6C3F1) received i.p. injections of N-nitrosodiethylamine (5mg/kg) or vehicle. Beginning at 5–7 weeks of age, mice were exposed to 0, 292, or 2056 ppm of PS-6 blend UG vapor for 6 h/day, 5 days/week for 16 weeks, 1 ppm ethinyl estradiol (EE2) in the diet, or 2056 ppm UG vapor and 1 ppm EE2 in the diet. Treatment with 2056 ppm UG but not 292 ppm UG increased relative liver weight, the number of macroscopic hepatic neoplasms, and the size and volume fraction of altered hepatic foci in N-nitrosodiethylamine-initiated mice. Treatment with 2056 ppm UG reduced relative uterus, ovary, and pituitary weights but did not change serum 17β-estra-1,3,5(10)-trien-3-ol, uterus peroxidase activity, or uterine cytoplasmic estrogen receptor levels. EE2 treatment reduced the number and size of altered hepatic foci in N-nitrosodiethylamine-initiated mice, caused weight loss, anestrus, vaginal keratinization, decreased uterine peroxidase activity, and decreased uterine cytoplasmic estrogen receptor levels. UG/EE2 co-treatment attenuated the weight loss, anestrus, and vaginal keratinization caused by EE2 treatment alone but dramatically increased the number and size of macroscopic hepatic neoplasms and the size and volume fraction of altered hepatic foci as compared to UG treatment alone. Thus, in this two-stage model of carcinogenesis (a) 2056 ppm UG had antiestrogenic effects, particularly with respect to pharmacological actions of EE2; (b) 2056 ppm UG but not 292 ppm UG acted as a liver tumor promoter; (c) EE2 inhibited liver tumor promotion; and (d) EE2 strongly potentiated liver tumor promotion by UG. These data demonstrate significant individual and interactive effects of UG vapor and estrogens in liver tumor promotion in female mice.

INTRODUCTION

The general public is routinely exposed to UG4 vapors in the environment, most commonly while pumping UG at service stations. Chronic exposure of both sexes of mice and rats to 67, 292, and 2056 ppm UG vapor induced liver tumors only in female mice and only at the highest exposure level (1). While UG has little or no genotoxic activity in a variety of short-term assays (2–4), exposure of DEN-initiated female mice to 2038 ppm UG vapor for 13 weeks modestly increased the size and volume fraction of hepatocellular foci (5). These findings, coupled with the mitogenic and cytochrome P450-inducing activity of UG in mice (5, 6), are consistent with the hypothesis that UG acts as a liver tumor promoter in female mice at high exposure levels. However, the reason for the sex- and species-specific hepatocarcinogenicity of UG and the relevance of this carcinogenic effect to human health are unclear.

A recent reexamination of tissue sections from the 2-year cancer bioassay of UG found a marked reduction in the severity of cystic hyperplasia in uteri of mice exposed to 2056 ppm UG as compared to controls (7). Moreover, uteruses from 35% of the mice exposed to 2056 ppm UG were atrophic, whereas none of the uteruses of control mice had this lesion. The authors suggested that the uterine changes and increased liver tumors at the high exposure level of UG may be related via changes in the hormonal environment (7). To pursue this question, we hypothesized that high concentrations of UG are antiestrogenic in mice and that liver tumor promotion by UG is secondary to antiestrogenicity. This hypothesis is based on the fact that estrogens are necessary for uterine growth, and antagonism of estrogenic actions might induce the aforementioned uterine changes. Moreover, EE2 treatment dramatically increased the growth of hepatocellular foci and/or the incidence of tumors in female mice initiated with various carcinogens (9–14). Thus, in contrast to the well-established liver tumor promoting activity of synthetic estrogens in rats (15–19), estrogens apparently inhibit liver tumor promotion in mice.

To determine if UG might promote female mouse liver tumors via an antiestrogenic mechanism, we used an initiation-promotion protocol to study (a) whether the medium and high exposure levels of UG from the cancer bioassay would cause liver tumor promotion and/or antiestrogenic effects in female mice; (b) whether estrogen treatment alone would suppress hepatocellular foci growth in DEN-initiated female mice; and (c) if estrogen supplementation would block liver tumor promotion in female mice also treated with UG vapor.

MATERIALS AND METHODS

Chemicals. PS-6 blend UG was provided by the American Petroleum Institute (Washington, DC) and was from the same lot used in the cancer bioassay (1). 2,4,6,7-[3H]17β-estradiol was obtained from NEN Research Products (Boston, MA). Unless otherwise specified, all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals. All experiments were conducted under NIH guidelines for the care and use of laboratory animals and were approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee. Virus-free male C3H/HeNCr1BR mice and female C57BL/6NCr1BR mice were obtained from Charles River (Raleigh, NC). After a 2-week acclimation period, the mice were bred to obtain the B6C3F1 offspring used in the studies described below.

Animal Treatments. At exactly 12 days of age, female B6C3F1 mice received i.p. injections of either 5.0 mg/kg DEN in 0.9% NaCl or 0.9% NaCl alone (7.1 ml/kg). The mice were weaned at 4–6 weeks of age. At 5–7 weeks of age, mice from the DEN initiation and NaCl control groups were each randomized by weight to one of several promotion groups (n = 12). Mice were exposed to 0, 292, or 2056 ppm (target concentrations) of wholly vaporized PS-6 blend UG for 6 h/day, 5 days/week, for 16 weeks. Exposure conditions were chosen to reproduce those used in the original bioassay (1). Exposures were routinely conducted from ~8:00 a.m.–2:00 p.m. on weekdays, including holidays. The chamber design, exposure generation system, and monitoring system were exactly as described previously (6) with chamber concentrations of UG determined hourly. The average daily chamber concentrations of UG during exposure periods were 2038 ± 56 ppm (99.1% of target) and 283 ± 15 ppm (96.9% of target) in the high and low concentration chambers, respec-
tively. Additional groups of mice were exposed to 1 ppm EE2 in the diet, or 1 ppm EE2 in the diet and 2056 ppm UG vapor as described above.

**Animal Husbandry.** Pregnant dams and postweaning B6C3F1 mice were housed individually in polystyrene cages on a cellulose bedding. Food (NIH-07 open formula diet; Ziegler Brothers, Gardners, PA) and filter-purified tap water were provided *ad libitum*. Mice were kept on a 12-h light-dark cycle, with the light period extending from 6 a.m. to 6 p.m. Temperature was maintained at 22 ± 2°C with relative humidity at 50 ± 10%.

During the promotion phase of the study, mice were housed in individual hanging stainless steel cages contained in a 1 m³ whole body inhalation chamber. Filter-purified tap water was available *ad libitum*, whereas food was only available during noninhalation exposure periods. EE2-supplemented diets and control diets (NIH-07 open formula diet) from the same lot were mixed and pelleted by Dyets, Inc. (Bethlehem, PA).

Clinical observations and body weights for individual mice were recorded weekly. The virus-free status of the mice was confirmed monthly by standard serological analysis performed on sentinel mice.

**Necropsy.** Approximately 20 h after the last inhalation exposure, mice were weighed, anesthetized with isoflurane, and euthanized by exanguination. Blood collected by cardiac puncture was allowed to clot for 0.5–1.0 h and then centrifuged to obtain serum. Livers were weighed and examined macroscopically. Macroscopic masses ≥1 mm in diameter were counted, and sections of some masses ≥4 mm were fixed in 10% neutral buffered formalin for processing to paraffin sections and microscopic examination. Sections of the left, median right, and right anterior lobes were fixed in formalin or frozen immediately in liquid nitrogen. Ovaries were dissected from surrounding tissue and weighed together. Uteri were trimmed of extraneous fat and weighed without expressing uterine fluid. One uterine horn was frozen immediately in liquid nitrogen, a section that included the uterine body and horn was fixed in 10% formalin, and the balance was weighed, pooled with uteris from 2 other mice (except for EE2-treated mice), and homogenized in 10 ms Tris (pH 7.4). The uterine homogenate was kept on ice for up to 6 h and then used to prepare a uterine extract in 0.5 M CaCl₂ by the method of Lytle and DeSombre (20). The remaining mouse carcass was fixed in 10% buffered formalin.

Forty-eight h after necropsy, the formalinized uteris were fixed in liver, ovaries, and uterus was replaced by 70% ethanol. Pituitaries were removed after formalin fixation for 2 months and weighed to the nearest 0.1 mg. All formalin-fixed tissues were embedded in paraffin, sectioned at 5 μm, stained with H & E, and examined by light microscopy.

**Preparation of Uterine Cytosol.** Uteri frozen at −70°C were thawed and homogenized (15 mg tissue/ml) with two 5-s bursts of a Tissumizer homogenizer (Tekmar Co., Cincinnati, OH) in ice-cold 10 mM Tris-1.5 mMEDTA-5 mM sodium molybdate-1 mM monothioglycol, pH 7.4 (21). The homogenate was centrifuged at 470 × g at 4°C for 10 min. The supernatant was removed and centrifuged at 20,000 × g at 4°C for 45 min. The supernatant (cytosol) was used for the estrogen receptor assay.

**Assays.** Unoccupied cytosolic estrogen receptors were measured using a modification of previously reported methods (21, 22). Briefly, aliquots (0.2 ml) of uterine cytosol were combined with [³H]17β-estradiol (final concentration, 10 nm) in the presence or absence of diethylstilbestrol (final concentration, 1 μM). Samples were incubated at 4°C for 18 h, and then 0.3 ml of 0.25% charcoal-0.025% dextran suspension was added. After a 15-min incubation on ice, samples were centrifuged at 10,000 × g for 3 min at 4°C. Radioactivity in the supernatant was quantitated by liquid scintillation counting. Specific binding was calculated from the difference in radioactivity between the incubation in the absence (total binding) and presence (nonspecific binding) of diethylstilbestrol.

**Protein was assayed with Coomassie-Plus Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin (Pierce) as a standard.**

**SDH activity in serum was assayed immediately after necropsy using a commercial kit (Sigma No. 50-UV) and a Roche Cobas Farra II clinical analyzer.**

**Uterine peroxidase activity was assayed in uterine extracts stored for 1–3 days at 4°C by the method of Lytle and DeSombre (20).** Preliminary studies indicated that uterine peroxidase activity did not change significantly when uterine extracts were stored for at least 11 days at 4°C. A unit was defined as the amount of enzyme required to produce an increase of 1 absorbance unit/ min under the assay conditions (20).

17β-Estradiol levels in serum stored for 6 months at −70°C were measured with the solid phase radioimmunoassay Coat-a-Count Estradiol (Diagnostic Products Corp., Los Angeles, CA) using a 3.5-h room temperature incubation. The percentage cross-reactivity of the antibody to EE2 is reported by the manufacturer to be 1.8%, whereas the intraassay and interassay variation are ~18 and 15%, respectively, at the relevant serum estrogen levels.

Frozen liver was sectioned at 5 μm and stained for glucose-6-phosphatase by the method of Wachstein and Meisel (23) and for GGT by the method of Rutenberg et al. (24).

**Quantitation of Altered Hepatic Foci.** Light microscopic examination of H & E-stained sections of gross hepatic masses indicated that they were large enough to be accurately examined. The largest AHF caused some compression of surrounding normal liver and thus could be considered borderline adenomas. However, all AHF were grouped together for the purposes of stereological analysis.

The total area of liver on an H & E-stained section was determined with an Image-1 image processing system (Universal Imaging Corp., West Chester, PA). Sections were examined, without knowledge of treatment, for the presence of AHF ≥10 cells in size, and foci were classified according to histopathological phenotype using standard criteria (25). The area of each focus was recorded and used to calculate the number and volume of foci according to the stereological method of Pugh et al. (26) using a focal profile cutoff with radius = 65 μm.

**Statistics.** All data are presented as mean ± SD. Data for control and DEN initiation groups treated with the same promotion regimen were compared by an unpaired, two-tailed t test and, if they were not significantly different for any of the promotion regimens, the data were pooled. Incidence data were evaluated by χ² analysis. Vaginal keratinization scores were evaluated by Fisher’s Exact Test using Bonferroni’s correction. Multiple comparisons were generally made by one-way analysis of variance followed by Dunn’s test if significant differences were found. Multiple comparisons of body weight gain beginning at week 4 were made using repeated measures analysis of variance. Because foci data were not normally distributed, these data were log transformed prior to analysis of variance. A value of 0.1 was substituted for zero values so that all foci data would be non-zero for log transformation. Differences were considered significant if P < 0.05.

**RESULTS**

**Body Weight.** A dietary concentration of 10 ppm EE2 was initially chosen for the EE2-treated groups because that was the dose reported by Lee et al. (8) to suppress foci growth in male mice. However, we observed a dramatic weight loss in mice treated with 10 ppm EE2 for 8 days (Fig. 1). While weight loss is an expected effect of estrogens in rodents (15, 18, 27–31), such profound weight loss was not deemed desirable. Thus, the 10 ppm EE2 diet was replaced with control diet for 8 days for both the EE2 and EE2/UG groups, during which time the body weights of these mice returned to control levels. When these groups were refed a diet of 1 ppm EE2, the mice treated with EE2 alone immediately lost ~30% of their body weight and then failed to gain weight at the rate of controls. The body weight of mice cotreated with EE2 and UG initially was similar to control but became decreased when these mice failed to gain weight at the rate of controls. Mice treated with 292 or 2056 ppm UG alone gained weight at the same rate as controls (Fig. 1). DEN-initiated mice exhibited similar body weight trends as shown in Fig. 1 for noninitiated mice, and this fact is reflected in the final body weights for these mice (Table 1).

**Organ Weights.** Relative organ weights, together with final body weights, are shown in Table 1. Treatment with 2056 ppm UG increased relative liver weight approximately 20% and decreased relative uterus, ovary, and pituitary weights approximately 40, 20, and 15%, respectively, irrespective of DEN treatment. Treatment with 292 ppm UG did not affect the weight of any of the organs examined except for a decrease in relative uterus weight in the control/UG 292 group that just reached statistical significance. EE2 treatment increased relative uterus weight and decreased relative ovary weight, as expected, and also increased relative liver weight and decreased rela-
Hepatic Preneoplastic Findings. Pale white neoplasms were observed macroscopically only in the livers of DEN-initiated mice. The quantitation of hepatic neoplasms in the different promotion groups is shown in Table 2. Treatment with 2056 ppm UG caused a 10.3-fold increase in the number of hepatic neoplasms, while treatment with 292 ppm UG or EE2 alone did not significantly affect the number of neoplasms. In contrast, EE2/UG cotreatment resulted in over a 60-fold increase in the number of hepatic neoplasms (Table 2).

To better quantify preneoplastic focus development, the number and size of AHF were determined. It was noted that 82 of 1080 total AHF (7.6%) appeared to protrude into the lumen of hepatic veins, which is common for DEN-initiated AHF in this model (32, 33). Approximately 90% of the foci were classified as basophilic, whereas the bulk of the remainder were mixed (basophilic and clear cell). A few small eosinophilic and clear cell foci were also observed. The different foci phenotypes were evenly distributed among the treatment groups, except for a greater proportion of mixed foci in the EE2/UG promotion group (data not shown). Since the vast majority of foci were basophilic, foci were grouped together for the purposes of stereological analysis. No AHF were observed in noninitiated mice. Treatment of DEN-initiated mice with 292 ppm UG did not significantly change the number, size, or volume fraction of AHF, whereas treatment with 2056 ppm UG caused 2.7- and 3.9-fold increases in size and volume fraction of AHF, respectively (Table 2). EE2 treatment substantially decreased the number, size, and volume fraction of AHF in DEN-initiated mice. In contrast, EE2/UG cotreatment resulted in dramatic 16-fold increases in focal size and volume fraction as compared to DEN-initiated controls (Table 2).

To determine if any of the promotion treatments induced AHF that were not detected by H & E staining, frozen liver sections from DEN-initiated mice (2-3 mice per group) were stained for glucose-6-phosphatase and GGT. Virtually all of the AHF detected by H & E staining of the frozen sections, regardless of treatment group, also stained positively for glucose-6-phosphatase (data not shown), which is typical of DEN-initiated foci in this two-stage model (14, 32). GGT-positive foci, which are observed in rats following DEN initiation and EE2 promotion (15-19), were not detected in any of the treatment groups (data not shown).

Reproductive Tract and Pituitary Pathology. At necropsy, the vaginas of EE2-treated mice were markedly dilated by a white lamellated mass approximately 5 mm in diameter. Macroscopic examination revealed that the mass represented a large accumulation of keratinized mass approximately 5 mm in diameter. Macroscopic examination of the frozen sections, regardless of treatment group, also stained negatively for glucose-6-phosphatase (data not shown), which is typical of DEN-initiated AHF in this model (32, 33). AHF (7.6%) appeared to protrude into the lumen of hepatic veins, which is common for DEN-initiated AHF in this model (32, 33).

Table 1

<table>
<thead>
<tr>
<th>DEN (+/-)</th>
<th>Promotion treatment</th>
<th>Final body wt (g)</th>
<th>Liver</th>
<th>Uterus</th>
<th>Ovaries (10 x %)</th>
<th>Pituitary (10^3 x %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>Control</td>
<td>31.1 ± 2.7</td>
<td>5.44 ± 0.34</td>
<td>0.57 ± 0.15</td>
<td>0.61 ± 0.06</td>
<td>11.9 ± 1.2</td>
</tr>
<tr>
<td>+</td>
<td>Control</td>
<td>31.7 ± 2.7</td>
<td>5.63 ± 0.42</td>
<td>0.50 ± 0.20</td>
<td>0.62 ± 0.11</td>
<td>12.0 ± 1.2</td>
</tr>
<tr>
<td>-</td>
<td>UG 292</td>
<td>31.6 ± 2.8</td>
<td>5.42 ± 0.46</td>
<td>0.42 ± 0.16</td>
<td>0.53 ± 0.06</td>
<td>11.2 ± 1.7</td>
</tr>
<tr>
<td>+</td>
<td>UG 292</td>
<td>30.6 ± 2.8</td>
<td>5.55 ± 0.37</td>
<td>0.46 ± 0.17</td>
<td>0.57 ± 0.09</td>
<td>11.4 ± 1.1</td>
</tr>
<tr>
<td>-</td>
<td>UG 2056</td>
<td>31.7 ± 2.7</td>
<td>6.39 ± 0.46</td>
<td>0.31 ± 0.10</td>
<td>0.50 ± 0.09</td>
<td>10.1 ± 1.6</td>
</tr>
<tr>
<td>+</td>
<td>UG 2056</td>
<td>31.1 ± 2.3</td>
<td>6.77 ± 0.54</td>
<td>0.32 ± 0.09</td>
<td>0.47 ± 0.06</td>
<td>10.3 ± 1.3</td>
</tr>
<tr>
<td>-</td>
<td>EE2</td>
<td>20.3 ± 1.2</td>
<td>6.48 ± 0.52</td>
<td>0.84 ± 0.09</td>
<td>0.22 ± 0.06</td>
<td>10.6 ± 1.0</td>
</tr>
<tr>
<td>+</td>
<td>EE2</td>
<td>20.4 ± 0.7</td>
<td>6.89 ± 0.74</td>
<td>0.78 ± 0.10</td>
<td>0.22 ± 0.04</td>
<td>11.0 ± 0.99</td>
</tr>
<tr>
<td>-</td>
<td>EE2/UG</td>
<td>27.2 ± 1.1</td>
<td>8.57 ± 0.98</td>
<td>0.68 ± 0.12</td>
<td>0.21 ± 0.03</td>
<td>10.0 ± 1.3</td>
</tr>
<tr>
<td>+</td>
<td>EE2/UG</td>
<td>25.2 ± 1.5</td>
<td>11.11 ± 1.11</td>
<td>0.70 ± 0.14</td>
<td>0.17 ± 0.02</td>
<td>10.7 ± 1.5</td>
</tr>
</tbody>
</table>

*See Fig. 1 legend for details.
+ +, with; -, without.
*Significantly different from noninitiated control.
*Significantly different from DEN-initiated control.
LIVER TUMOR PROMOTION BY UNLEADED GASOLINE

Female B6C3F1 mice received i.p. injections of DEN (5 mg/kg) at 12 days of age. Beginning at 5–7 weeks of age, mice were treated with 0 (control), 292, or 2056 ppm UG vapor for 6 h/day and 5 days/week for 16 weeks, 1 ppm EE2 in the diet, or 1 ppm EE2 in the diet and 2056 ppm UG vapor for 16 weeks. Macroscopic hepatic masses ≥1 mm at necropsy were counted. The area occupied by AHF on cross sections of liver was used to estimate the volume for 6 h/day and 5 days/week for 16 weeks, 1 ppm EE2 in the diet, or 1 ppm EE2 in the diet and 2056 ppm UG vapor for 16 weeks. Macroscopic vaginal keratinization was scored from Fig. 1 legend for details. * Significantly different from noninitiated control group.

### Table 2. Number of gross hepatic masses and parameters of altered hepatic foci in DEN-initiated mice

<table>
<thead>
<tr>
<th>Promotion treatment</th>
<th>Macromosaic hepatic neoplasms (no. ± 1 mm)</th>
<th>Altered hepatic foci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (no./liver)</td>
<td>Mean volume (no./liver)</td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.8</td>
<td>353 ± 210</td>
</tr>
<tr>
<td>UG 292</td>
<td>1.4 ± 1.6</td>
<td>485 ± 257</td>
</tr>
<tr>
<td>UG 2056</td>
<td>7.2 ± 4.6*</td>
<td>533 ± 202</td>
</tr>
<tr>
<td>EE2</td>
<td>0.8 ± 1.2</td>
<td>228 ± 184*</td>
</tr>
<tr>
<td>EE2/UG</td>
<td>4.73 ± 9.9*</td>
<td>507 ± 217</td>
</tr>
</tbody>
</table>

* See Fig. 1 legend for details.

### Table 3. Summary of pathological changes in female mouse reproductive tract

<table>
<thead>
<tr>
<th>DEN (+/−)</th>
<th>Promotion treatment</th>
<th>Vaginal keratinization severity (no. of mice)</th>
<th>Corpora lutea</th>
<th>Cystic glands in uteri</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Moderate</td>
<td>Severe</td>
<td>Incidence</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12/12</td>
</tr>
<tr>
<td>+ Control</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12/12</td>
</tr>
<tr>
<td>UG 292</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12/12</td>
</tr>
<tr>
<td>UG 2056</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12/12</td>
</tr>
<tr>
<td>+ UG 2056</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>12/12</td>
</tr>
<tr>
<td>EE2</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12/12</td>
</tr>
<tr>
<td>+ EE2</td>
<td>1</td>
<td>1</td>
<td>10d</td>
<td>0/12d</td>
</tr>
<tr>
<td>− EE2/UG</td>
<td>4</td>
<td>6</td>
<td>2e</td>
<td>3/12e</td>
</tr>
<tr>
<td>+ EE2/UG</td>
<td>6</td>
<td>2</td>
<td>4de</td>
<td>0/12de</td>
</tr>
</tbody>
</table>

* See Fig. 1 legend for details.

### DISCUSSION

A major objective of this study was to test the hypothesis that UG causes antiestrogenic effects in female B6C3F1 mice under the same exposure conditions, albeit of shorter duration, as those that caused liver tumors in a 2-year cancer bioassay. Several findings were consistent with this hypothesis: (a) treatment of adult female mice with 2056 ppm UG for 16 weeks decreased relative uterine estrogen receptor levels. Neither 292 nor 2056 ppm UG significantly decreased basal uterine estrogen receptor activity in the present study, whereas EE2 treatment unexpectedly caused an 80% decrease in uterine estrogen activity (Fig. 3). EE2/UG cotreatment suppressed estrogen receptor activity to the same extent as EE2 alone (Fig. 3).

Since suppression of estrogen receptors is often associated with antiestrogenticity in mice (21, 38), we determined whether UG treatment affected unoccupied uterine estrogen receptor levels. Neither 292 nor 2056 ppm UG significantly changed uterine estrogen receptor levels, whereas EE2 treatment alone and EE2/UG cotreatment significantly suppressed estrogen receptor levels in DEN-initiated mice (Fig. 4).

### Acknowledgments

This work was supported by a grant from the California Air Resources Board (grant 92-252). The authors are indebted to the staff of the National Toxicology Program, especially T. H. Shih and J. M. Smith, for their continuing support of the rodent cancer bioassay. They are grateful to Dr. M. H. Heindel, University of California School of Medicine, for advice on statistical analysis.
ppm UG also significantly reduced ovary and pituitary weights, but these effects were relatively small and have many possible explanations besides antiestrogenic activity; (b) co-treatment of female mice with 2056 ppm UG and EE2 partially reversed the body weight suppression caused by EE2 alone. Exogenous estrogen treatment is well known to suppress body weight in rats and mice, and this effect is mediated at the level of the hypothalamus (15, 18, 27–31). Thus, the partial reversal of EE2-induced body weight suppression by UG represents an antiestrogenic effect of UG on exogenous EE2; (c) the ovaries of several mice cotreated with 2056 ppm UG and EE2 contained corpora lutea, whereas corpora lutea were uniformly absent in ovaries of mice treated with EE2 alone. Corpora lutea are indicative of ovulation, and the complete cessation of ovulation (anestrus) is an expected effect of pharmacological levels of EE2 (39). Thus, the reversal of EE2-induced anestrus by UG cotreatment represents an antiestrogenic effect of UG on exogenous EE2; and (d) a finding indicative of antiestrogenicity of UG was the decreased severity of vaginal keratinization in EE2/UG cotreated mice relative to mice treated with EE2 alone.

In contrast to the above findings, several pieces of data were not supportive of the hypothesis that high concentrations of UG are antiestrogenic in mice. UG treatment did not decrease uterine cytosolic estrogen receptor levels or decrease serum 17β-estradiol levels. Such effects might be expected if potential antiestrogenicity of UG involved down-regulation of estrogen receptor levels (21, 38) or enhanced clearance of estrogens (40), respectively. UG treatment also did not significantly decrease basal uterine peroxidase activity, which might be expected if UG antagonized endogenous estrogens (20, 37). UG treatment actually increased uterine peroxidase activity, which was unexpected given that short-term estrogen treatment is well known to induce this activity (41). The EE2-induced decrease of uterine peroxidase activity may have been a consequence of long-term, high-dose exposure to EE2. Consistent with this idea, 17β-estradiol treatment of rabbits was reported to cause much greater stimulation of uterine peroxidase activity after 1 week than after 4 or 8 weeks of continuous treatment (42). UG treatment of mice did not cause obvious histopathological changes in several organs that either produce or respond to endogenous estrogens, including the uterus, ovary, and pituitary.

To summarize the data on antiestrogenicity, subchronic treatment of female mice with 2056 ppm UG vapor had several antiestrogenic effects, including decreasing relative uterus weight and partially reversing EE2-induced body weight suppression, anestrus, and vaginal keratinization. However, UG treatment did not significantly decrease serum 17β-estradiol levels, uterine peroxidase activity, or uterine cytosolic estrogen receptor levels or induce histopathological changes in the female mouse reproductive tract. Taken together, these data provide relatively strong support for an antiestrogenic effect of UG on exogenous EE2 but relatively weak support for an antiestrogenic effect of UG on endogenous estrogens. While the end points available to evaluate the latter were limited in sensitivity due to our desire to mimic the conditions of the cancer bioassay (1), e.g., the use of randomly cycling mice (43–45), the fact remains that not all the data were consistent with antiestrogenicity of UG. Further experiments will therefore be needed to evaluate the potential antiestrogenicity of UG.

With regard to liver tumor promotion, treatment with 2056 ppm UG for 16 weeks increased the size and volume of AHF by 3–4 fold in DEN-initiated female mice. This effect was almost identical to the liver tumor promoting effect of UG exposure for 13 weeks in the same two-stage model (5). Exposure to 292 ppm UG did not increase number or size of AHF, thus demonstrating a “no-effect” level for liver tumor promotion by UG in this model. This lack of promotion by 292 ppm UG is consistent with the lack of mitogenic or hyperplastic effects of this exposure level in the female B6C3F1 mouse (6, the present study). This exposure level of UG also had virtually no effect on the parameters of antiestrogenicity, which is consistent with the hypothesis that an antiestrogenic level of UG may be necessary for liver tumor promotion in female mice.

A keystone of the hypothesis that UG promotes liver tumors in female mice secondary to antiestrogenicity is the concept that estrogens normally act to suppress liver tumor promotion in mice (11, 46). This concept is inferred from the fact that ovariectomy enhances liver tumor promotion in female mice (9–14), even though ovariectomy has other effects besides decreasing circulating estrogen levels (12). Treatment of several strains of male mice with 10 ppm EE2 in the diet was shown to suppress the number and size of AHF in a two-stage model of carcinogenesis (8), thus providing direct evidence that estrogens...
suppress liver tumor promotion in male mice. The comparable experiment had not, to our knowledge, been conducted in female mice. Thus, our finding that 1 ppm dietary EE2 decreased the number, size, and volume of AHF in DEN-initiated female mice is a novel result and establishes antiestrogenicity as a plausible mechanism of chemically induced liver tumor promotion in female mice. However, since 1 ppm EE2 also suppressed body weight, it remains to be shown whether or not estrogens inhibit liver tumor promotion in mice in the absence of weight loss. Caloric restriction, which is responsible for at least the initial weight loss in estrogen-treated animals (27, 31), has long been known to inhibit liver tumor development in mice (47). Ovariectomy of female mice increases body weight and hepatocarcinogenesis relative to intact controls (12-14). However, differences in body weight are unlikely to be the only factor affecting hepatocarcinogenesis in the present experiment since DEN-initiated, EE2/UG cotreated mice had lower body weights but greatly increased hepatic focal growth relative to DEN-initiated controls.

An unexpected finding was the dramatic potentiation of UG-induced liver tumor promotion by cotreatment with EE2. It was reasoned that if UG-induced liver tumor promotion resulted from antiestrogenic effects, then supplementation of these mice with estrogen would block liver tumor promotion. EE2 was chosen as opposed to 17β-estradiol since EE2 is relatively resistant to first-pass metabolism (39, 48) and thus could be administered conveniently in the diet. If it is assumed that EE2-treated mice consumed 2 g of feed per day, then the daily dose of EE2 was ~100 µg/kg, which is up to 200 times the daily dose of EE2 in humans taking oral contraceptives (39, 48). Such pharmacological levels of estrogen may have qualitatively different effects on liver tumor promotion than physiological levels of estrogen, in analogy with the unexpected suppression of uterine peroxidase by EE2 noted above. Alternatively, there may be qualitative differences between natural and synthetic estrogens with regard to liver tumor promotion (18). Thus, while the failure of EE2 supplementation to block liver tumor promotion in UG-treated mice does not support the overall hypothesis, it does not necessarily discount the hypothesis. The strong potentiation of UG-induced tumor promotion by EE2 cotreatment is itself an interesting chemical interaction that warrants further study.

In conclusion, treatment of female mice with UG vapor for 16 weeks under exposure conditions identical to those that induced liver tumors in female mice in a chronic bioassay (1) was shown to have some antiestrogenic effects, but these effects were mostly on pharmacological actions of exogenous EE2. Further study will be needed to determine if antiestrogenicity of UG is evident at physiological levels of estrogens. In the context of a two-stage model for carcinogenesis, these data also (a) establish a no-effect level for liver tumor promotion by UG; (b) provide direct evidence that an estrogen suppresses liver tumor promotion in female mice; and (c) demonstrate a dramatic potentiation of UG-induced liver tumor promotion by EE2 in female mice. These data reveal novel individual and interactive effects of UG vapor and estrogens in mouse liver tumor promotion.

ACKNOWLEDGMENTS

We gratefully acknowledge Paul Ross and Ardie James for coordinating animal care and inhalation exposures, respectively, and Mike Judge, Carol Bobbit, Kathy Bragg, Elizabeth Humphrey, Richard Masney, Mary Morris, Corrie Dunn, Otis Lyght, Delorise Williams, and Vonda Teets for technical assistance. We also thank Dr. Derek Janszen for statistical advice, Drs. Owen Moss and James Popp for helpful discussions, and Drs. Byron Butterworth and Rich Miller for critical review of the manuscript.

REFERENCES


Interactive Effects of Unleaded Gasoline and Estrogen on Liver Tumor Promotion in Female B6C3F₁ Mice

Andrew M. Standeven, Douglas C. Wolf and Thomas L. Goldsworthy


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/5/1198