Promotion of Hepatic Preneoplastic Lesions in Male B6C3F1 Mice by Unleaded Gasoline

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In previous studies, unleaded gasoline (UG) vapor was found to be a liver tumor promoter and hepatocarcinogen in female mice, but UG was not a hepatocarcinogen in male mice. However, UG vapor had similar transient mitogenic effects in nonlesioned liver of both male and female mice under the conditions of the cancer bioassay. We used an initiation-promotion protocol to determine whether UG vapor acts as a liver tumor promoter in male mice and to examine proliferative effects that may be critical to tumor development. Twelve-day-old male B6C3F1 mice were injected with N-nitrosodimethylamine (DEN; 5 mg/kg, intraperitoneally) or vehicle. Starting at 5–7 weeks of age, mice were exposed by inhalation 6 hr/day, 5 days/week for 16 weeks to 0 or 2046 ppm of PS-6 blend UG. UG treatment caused a significant 2.3-fold increase in the number of macroscopic hepatic masses in DEN-initiated mice, whereas no macroscopic masses were observed in non-initiated mice. Altered hepatic foci (AHF), which were predominantly basophilic in phenotype, were found almost exclusively in DEN-initiated mice. UG treatment significantly increased both the mean volume (threefold) and the volume fraction (twofold) of the AHF without increasing the number of AHF per unit area. UG also induced hepatic pentoxysresorulin-O-dealkylase (PROD) activity, a marker of CYP2B, by more than 12-fold over control with or without DEN cotreatment. To study hepatocyte proliferative effects of UG, we treated mice with 5-bromo-2’-deoxyuridine (BrdU) via osmotic pump for 3 days before necropsy and measured hepatocyte BrdU labeling index (LI) in AHF and nonlesioned liver. UG did not significantly affect BrdU LI in nonlesioned liver. However, hepatocyte LI in AHF was about 30% higher in DEN/UG-treated mice relative to mice treated with DEN alone. These data show that UG vapor promotes AHF in male mice and that liver tumor promotion is associated with a selective increase in hepatocyte proliferation in AHF. UG acts as a liver tumor promoter in both male and female mice, and these findings contrast with the lack of hepatocarcinogenicity of UG in male mice in a cancer bioassay. Key words: altered hepatic foci, cell proliferation, liver, gasoline, tumor promotion. Environ Health Perspect 103:696–700 (1995)

Enormous quantities of unleaded gasoline (UG) vapor are released into the air each year during transfer of UG to consumers. The general public is most commonly exposed to UG in the form of vapors that evaporate during refueling vehicles at service stations (1). The health risk of such intermittent, low-dose exposure to UG vapor is difficult to assess. In a cancer bioassay, a relatively high exposure level of UG vapor (2056 ppm, 6 hr/day, 5 days/week) but not lower levels (67 or 292 ppm UG) increased the incidence of hepatocellular tumors in female B6C3F1 mice (2). In contrast, the incidence of hepatocellular tumors in male B6C3F1 mice was not increased at any exposure level of UG (2,3). This sex-specific hepatocarcinogenic effect of UG in mice has been the subject of several mechanistic studies. Tilbury et al. (4) showed that 2056 ppm UG vapor induced hepatocyte proliferation without evidence of hepatotoxicity in both male and female B6C3F1 mice only in the first week of 13 weeks of intermittent exposure. The lack of sustained hepatocyte proliferation and the induction of proliferation in both sexes of mice make mitogenesis an unlikely explanation for the sex-specific induction of liver cancer by UG. Nonetheless, many hepatic mitogens are known mouse liver tumor promoters (5), and UG vapor was subsequently shown to be a weak liver tumor promoter in female B6C3F1 mice initiated with N-nitrosodimethylamine (DEN) (6). UG, including the specific blend used in the cancer bioassay, has shown little or no genotoxic activity in a variety of assays (7–9).

Recently, MacGregor et al. (10) reported that 2056 ppm of UG caused a high incidence of uterine atrophy in female mice in the cancer bioassay, suggesting that UG may disrupt the hormonal balance of female mice. We hypothesized that UG might have antiestrogenic effects and that estrogen antagonism might be causally related to liver tumor promotion in female mice (11) because estrogens and/or ovarian factors are known to inhibit liver tumor promotion in mice (12–18). Indeed, intermittent exposure to 2056 ppm but not to 292 ppm of UG vapor for 16 weeks promoted the growth of preneoplastic lesions and decreased relative uterine weight in DEN-initiated female mice (11). UG also antagonized several pharmacological effects of exogenous ethinyl estradiol (17). UG by intragastric intubation caused a dose-dependent increase in estrogen metabolic capacity in hepatocytes isolated from female mice (19), thus suggesting a mechanism by which UG might antagonize estrogen. Although UG antagonizes pharmacological levels of estrogen, a clear causal relationship between antagonism of endogenous estrogens and liver tumor promotion by UG has not been established.

An assumption of this antiestrogenic hypothesis of UG carcinogenesis is that antagonism of the presumably low estrogen levels in male mice would be inconsequential with regard to liver tumor promotion, thus accounting for the lack of hepatocarcinogenicity of UG in male mice. Moreover, the strong liver-tumor-promoting effect of androgens might be expected to be the predominant hormonal influence in male mice (12,13,17). However, given the similar acute mitogenic response of male and female mouse liver to UG vapor and the potential relationship of mitogenicity to tumor promotion, it was of interest to test whether UG acted as a liver tumor promoter in male mice. In the present study, the possible tumor-promoting activity of UG in male mice was tested in the same initiation–promotion model in which liver tumor promotion by UG was demonstrated in female mice (5,11). In addition, the cellular basis of such promoting activity was examined by analysis of hepatocellular proliferation in altered hepatic foci (AHF) and surrounding nonlesioned liver.

Materials and Methods

PS-6 blend UG was provided by the American Petroleum Institute and was from the same lot used in the cancer bioassay. Unless otherwise specified, all other chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri). All experiments were conducted under NIH guidelines for the care and use of laboratory animals and were approved by the CIIT Institutional Animal Care and Use Committee. Male C3H/HeNc1BR mice and female C57BL/6Nc1BR mice free of common murine pathogens were obtained from Charles River Breeding Labs (Raleigh, North Carolina) and acclimated for 10 days. Mice were housed individually in polystyrene cages on α-cellulose bed-

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ding in a temperature- and humidity-con-
trolled room. Mice were kept on a 12-hr
light–dark cycle, with the light period
extending from 0600 hr to 1800 hr. Food
(NIH-07 open formula diet; Ziegler
Brothers, Gardners, Pennsylvania) and fil-
ter-purified tap water were provided ad
libitum. The mice were then bred, and the
resulting B6C3F1, offspring were treated as
described below.

At exactly 12 days of age, we injected
male B6C3F1 mice intraperitoneally with 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 and housed individually as described
above.

At 5–7 weeks of age, the B6C3F1 mice
from the DEN initiation and NaCl control
groups were separately randomized by
weight, assigned to one of two groups (n =
12), and transferred to individual hanging
stainless-steel cages contained in a 1-m
whole-body inhalation chamber. The mice
were exposed to 0 or 2046 ppm (target con-
centration) of wholly vaporized PS-6 blend
UG for 6 hr/day, 5 days/week, for 16 weeks.

Observations and Exposures

Exposures were routinely conducted from
0800 hr to 1400 hr on weekdays, including
holidays. The chamber design, exposure gen-
eration system, and monitoring system were
exactly as described previously (5), and
chamber concentrations of UG were deter-
mined hourly. Average daily chamber con-
centrations of UG ranged from 1741 to
2146 ppm, with a mean and SD of 2046 ±
63 ppm (99.5% of target level). Filter-puri-
tified tap water was available ad libitum,
whereas food was only available during non-

Table 1. Final body weights, relative liver weight, hepatic PROD activity, and serum SDH activity in male
B6C3F1 mice

| Treatment | Final body weight (g) | Liver weight (% of body weight) | Hepatic PROD activity (μmol/min/mg) | Serum SDH activity (U/L) |
|-----------|----------------------|-------------------------------|-----------------------------------|------------------------|
| Saline/control | 35.2 ± 2.3          | 5.03 ± 0.36                   | 185.5 ± 2.9                      | 24.5 ± 4.0             |
| Saline/UG | 34.7 ± 2.1          | 5.62 ± 0.81                   | 215.0 ± 19.9*                    | 30.8 ± 6.0             |
| DEN/control | 35.6 ± 2.4          | 6.36 ± 0.98                   | 11.2 ± 2.2                       | 27.3 ± 4.1             |
| DEN/UG | 36.0 ± 2.8          | 8.30 ± 0.88**                  | 172.0 ± 27.4**                   | 35.0 ± 8.7             |

Abbreviations: PROD, pentoxysorufin-C-dealkylase; SDH, sorbitol dehydrogenase; DEN, N-nitroso-
diethylamine; UG, unleaded gasoline.

Male B6C3F1 mice were injected with DEN (5 mg/kg, intraperitoneally) or vehicle (saline) at 12 days of age.
Beginning at 5–7 weeks of age, mice were treated with 0 (control) or 2046 ppm UG vapor for 6 hr/day
and 5 days/week for 16 weeks.

Values are the means ± SD of 11–12 mice.

Values are the means ± SD of four microsomal samples that were each pooled from three mice.

*p < 0.05 as compared to saline/control group.

*p < 0.05 as compared to DEN/control group.

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

| Promotion treatment | No. of gross hepatic masses ≥1 mm | Density (No./liver) | Mean volume (103 x mm³) | Volume fraction (%) |
|---------------------|-----------------------------------|--------------------|------------------------|---------------------|
| Saline/control      | 0                                 | 0                  | 0                      | 0                   |
| Saline/UG           | 0                                 | 0                  | 0                      | 0                   |
| DEN/control         | 233.3 ± 13.6                      | 802 ± 419          | 292.7 ± 210.0          | 9.51 ± 5.72*        |
| DEN/UG              | 52.9 ± 20.1                       | 725 ± 259          | 851.9 ± 512.1          | 17.75 ± 8.16        |

Abbreviations: DEN, N-nitrosodiethylamine; UG, unleaded gasoline.

Male B6C3F1 mice were injected with DEN (5 mg/kg, intraperitoneally) at 12 days of age. Beginning at
5–7 weeks of age, mice were treated with 0 (control) or 2046 ppm UG vapor for 6 hr/day and 5 days/week
for 16 weeks.

*Assumes 1 liver = 1 cm³.

*Only one altered hepatic focus was detected in one animal of each of these groups. Thus, calculation of
focal parameters is omitted for these groups.

Values are the means ± SD of 11–12 mice.

*p < 0.05 as compared to DEN control.
We compared mean values for control and DEN-initiated mice to the corresponding UG-treated group by an unpaired, two-tailed t-test. To fulfill the requirement for homogeneity of variance, foci data were log transformed before statistical analysis. Differences were considered significant at \( p < 0.05 \).

**Results**

Inhalation exposures were not begun until the mice were 5–7 weeks old because that is the age at which exposures were begun in the cancer bioassy of UG. Treatment with DEN, UG, or the combination did not significantly affect body weight relative to controls at any time point (data not shown), and no adverse clinical signs were observed. One DEN/UG-treated mouse died as a result of a cage accident.

Treatment with UG increased liver weight with respect to controls for both noninitiated and DEN-initiated mice (Table 1). UG-induced hepatomegaly was greater in DEN-initiated mice, evidently due to the presence of numerous pale, white lesions in that group. DEN-initiated mice not exposed to UG had approximately half as many macroscopic neoplasms as in the DEN/UG group, and no neoplasms were observed grossly in initiation control mice (Table 2). Testicular weight and pituitary weight were not significantly affected by UG exposure (data not shown).

Induction of hepatic PROD activity, which is catalyzed principally by CYP2B (23,24), has been correlated with liver tumor promotion in rodents (25,26). Because hepatic PROD activity was induced in female mice exposed to 2056 ppm UG for 13 weeks (5), the possible effect of UG on hepatic PROD activity in male mice was determined. UG induced hepatic PROD by about 12-fold in uninitiated mice and by about 16-fold in DEN-initiated mice relative to corresponding controls (Table 1).

Serum SDH activity, a marker of hepatic necrosis, was not significantly elevated by UG treatment (Table 1), which is consistent with the lack of hepatotoxicity in male or female mice exposed to up to 2056 ppm UG for 13 weeks (4).

Tests and pituitaries of DEN, UG, and DEN/UG-treated mice were histologically indistinguishable from those of control mice. Modest centrilobular hepatocyte swelling was observed in H&E-stained liver sections from mice treated with 2056 ppm UG with or without DEN treatment; however, no hepatic necrosis was observed. In DEN-initiated mice, a large number of AHF were observed in H&E liver sections. Only one AHF was found in one animal in each of the noninitiated groups. Approximately 90% of the AHF were basophilic, and the majority of the remainder were mixed (basophilic/clear cell). Because the vast majority of AHF were basophilic, AHF were grouped together for the purpose of stereological analysis. As shown in Table 2, UG treatment increased the mean volume and volume fraction occupied by AHF by threefold and twofold, respectively. While this may seem to be a subtle effect, it is worth noting that the AHF in the DEN/control group were clearly at an advanced stage of development as judged by the relatively large mean volume and volume fraction occupied by AHF (Table 2). Thus, a twofold increase in volume fraction when volume fraction was already near 10% in the DEN/control group represents a highly biologically significant increase. The number of AHF per unit area, however, was not increased by UG treatment in DEN-initiated mice (Table 2).

To determine whether hepatocyte proliferation rates differed between treatment groups, mice were treated with BrdU via osmotic pumps for 3 days before necropsy, and BrdU incorporation was evaluated immunohistochemically in liver sections. UG did not increase nonlesioned hepatocyte LI in initiation control or DEN-initiated mice (Table 3). The hepatocyte LI of AHF was substantially greater than that of nonlesioned liver, as expected (27–29). The mean hepatocyte LI of AHF from DEN/UG-treated mice was 29% greater (\( p < 0.01 \)) than the mean hepatocyte LI of AHF from mice treated with DEN alone (Table 3). For these data, the hepatocyte LI of all the AHF in a given mouse were averaged together, and the value shown in Table 3 is the grand mean of all the mice in a group. Since different numbers of AHF were counted for each mouse, this value could be unduly influenced by mice with relatively few AHF. Thus, we also calculated the mean LI of the aggregate of UG-treated AHF (36.9 ± 7.6%, \( n = 63 \)).

![Figure 1](image-url) **Figure 1.** Size class of distribution of altered heptic foci (AHF) in N-nitrosodimethylamine (DEN)-initiated mice. Male B6C3F1 mice were injected intraperitoneally with DEN (5 mg/kg) or saline. Beginning at 5–7 weeks of age, mice were exposed to 0 or 2046 ppm of unloaded gasoline (UG) vapor for 6 hr/day, 5 days/week, for 16 weeks. The number of cells in all AHF ≤0.85 mm in diameter was determined on 5'-bromodeoxyuridine-stained liver sections (\( n = 20 \)), and the AHF were grouped into size classes as shown. The number of AHF in each size class is given as a percentage of the total counted, which was 99 and 63 in the DEN/control and DEN/UG groups, respectively.
activity, which fits with the correlation of CYP2B induction and the phenomenon of liver tumor promotion in rodents (25,26).

The liver-tumor-promoting activity of UG in male mice in this study appears to be at odds with the lack of carcinogenic activity in male mice in the cancer bioassay of UG (2). There are several potential reasons for this apparent discrepancy. First, the infant mouse initiation–promotion model used to detect the tumor promotion effect of UG may be more sensitive than the cancer bioassay, and only relatively robust promotional effects in the infant mouse model predict promotion activity in longer-term studies. For example, under identical exposure conditions, UG induced a fourfold increase in volume of AHF in female B6C3F1 mice (11) versus a twofold increase in male mice in the present study; and UG induced liver tumors in female and not in male mice in the cancer bioassay (2). However, the quantitative difference in UG promotion activity between male and female mice most likely is, in part, a consequence of the 16-week time point chosen for analysis. AHF in the DEN/control group were about fourfold larger in male than in female mice at this time point (11), which is consistent with the well-established faster growth of AHF in male mice in the infant mouse model (28,29). Thus, it would have been relatively difficult to demonstrate the same fourfold increase in size of AHF in male versus female mice at the 16-week time point. Nevertheless, these data caution against the use of sensitive tumor promotion models to predict cancer bioassay outcomes.

A second possible explanation for why UG acted as a liver tumor promoter in the present study but was not hepatocarcinogenic in male mice in the chronic bioassay is that the biology of DEN-initiated foci may be significantly different from the biology of the spontaneous foci that UG presumably acted upon in the chronic bioassay. It is well known that the phenotypes and genotypes of AHF are heterogeneous, even among morphologically similar AHF (17,31,32). DEN is a potent mutagen, and its administration to mice yields a spectrum of AHF phenotypes that may not be the same ones that progressed to liver tumors in the cancer bioassay of UG (17,33).

A third potential explanation for the apparent discrepancy between the present study and the results of the cancer bioassay of UG is that some factor(s) may be lacking in males, or present in females, that allows growth and progression of UG-promoted AHF to hepatic tumors selectively in females. For example, the particular hormonal milieu of the female mouse may have favored the progression of the types of spontaneous AHF promoted by UG in the cancer bioassay.

A novel finding in the present study was the small (-30%) but highly significant increase in hepatocyte LI in basophilic AHF from DEN-initiated, UG-treated mice relative to AHF from mice treated with DEN alone. UG did not increase the proliferative rate of surrounding nonlesioned hepatocytes, which is consistent with previous data showing that UG-induced hepatocyte proliferation disappeared after the first week of chronic, intermittent exposure (4). We are aware of only one other report (34) in which a mouse liver tumor promoter was shown to selectively increase the proliferative rate of AHF in male mice. In that study, phenobarbital selectively increased the LI of eosinophilic and not basophilic AHF but concomitantly suppressed LI in nonlesioned hepatocytes in female C3H mice (34). In rats, phenobarbital increased the number and size of DEN-initiated γ-glutamyl transpeptidase-positive nodules but did not change the average LI of these nodules (32). In another study, the LI of γ-glutamyl transpeptidase-positive hepatocytes as well as the LI of nonlesioned hepatocytes was increased by several liver tumor promoters, including phenobarbital, cyproterone acetate, and nafenopin (27).

The fact that AHF were larger, on average, in UG-treated mice might be a consequence of either a greater cellular birth rate, a decreased cellular death rate, or both, in these focal hepatocytes (6). The finding of a higher LI in AHF of UG-treated mice suggests that a greater cellular birth rate in these AHF at least contributed to promotion by UG. However, the molecular mechanism behind this increased proliferative rate remains to be determined. We propose that UG might have antiestrogenic effects in female mice and that estrogen antagonism might secondarily lead to liver tumor promotion (11,19). The molecular basis for the inhibitory effect of estrogen on promotion in mice is not known but has been observed in both male and female mice (13,14,18,19). The antiestrogenic hypothesis seems less plausible in male mice, which would be expected to have lower endogenous levels of estrogen for the UG to act upon. Indeed, we did not detect any effect of UG on testes or pituitary of male mice, whereas UG decreased ovarian and pituitary weight in female mice (11). However, we cannot rule out the possibility that UG-induced liver tumor promotion in our two-stage model follows a similar antiestrogenic mechanism in both sexes of mice. Clearly, more studies are needed to define the biochemical/molecular mechanism of UG-induced tumor promotion in both sexes of mice.

It is noteworthy that wholly vaporized UG, which has the composition of liquid gasoline, was used in both the cancer bioassay of UG (2) and the present liver tumor promotion study of UG, whereas most human exposure to UG involves UG vapor. The low boiling-point components of liquid UG are disproportionately represented in UG vapor (1,35). However, high boiling-point components of UG, which account for the bulk of the hepatic mitogenic and P450-inducing activity of liquid UG, are present in wholly vaporized UG (36). Thus, if such hepatic activities are causally related to liver tumor promotion by UG, the results of this tumor promotion study and the cancer bioassay of UG may well overstate human liver cancer risk from UG exposure.

In summary, we have shown that UG promotes the development of DEN-initiated AHF in male mice under exposure conditions similar to those that failed to increase liver tumor incidence in a cancer bioassay of UG. Promotion of DEN-initiated AHF in male mice was associated with a selective increase in proliferation of focal hepatocytes. Further studies are needed to define the mechanism of growth stimulation of hepatic preneoplastic lesions by UG and elucidate the reason such stimulation results in hepatic neoplasia selectively in female mice. Our findings illustrate that tumor-promoting activity does not necessarily predict carcinogenicity and that analysis of chemically induced hepatocyte proliferation in nonlesioned liver does not necessarily predict induced proliferation in preneoplastic lesions.

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