Promoting Effects of Unleaded Gasoline and 2,2,4-Trimethylpentane on the Development of Atypical Cell Foci and Renal Tubular Cell Tumors in Rats Exposed to N-Ethyl-N-hydroxyethylNitrosamine

Brian G. Short,1 William H. Steinhagen, and James A. Swenberg

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

ABSTRACT

Unleaded gasoline (UG), a nongenotoxic kidney carcinogen in male, but not female, F344 rats or either sex of mice, and 2,2,4-trimethylpentane (TMP), a representative nongenotoxic isoparaaffinic component of UG, were tested for potential promoting and cocarcinogenic effects in a kidney initiation-promotion model. The promotion study was conducted with 305 male and 305 female F344 rats fed 170 ppm N-ethyl-N-hydroxyethylnitrosamine in the drinking water for 2 weeks and then inhalation exposed to 0, 10, 70, or 300 ppm UG or 50 ppm TMP for 24 or 59 to 61 weeks. In a sequence reversal study, 390 male F344 rats were inhalation exposed to 0, 10, 70, or 300 ppm UG or 50 ppm TMP for 24 weeks, followed by 170 ppm N-ethyl-N-hydroxyethylnitrosamine in the drinking water during weeks 28 to 30, and killed at weeks 65 to 67. Renal neoplastic lesions were classified as atypical cell foci (ACF) and renal cell tumors (RCT). In the hydrocarbon promotion study, dose related increases were observed in the incidence of ACF in male rats promoted with UG or 50 ppm TMP for 24 or 60 weeks. A significant linear trend in the incidence of RCT was observed in male rats promoted with UG for 24 weeks. The incidence of ACF or RCT was not elevated in female rats promoted with UG or TMP. In the sequence reversal study, a slight increase in ACF was demonstrated in male rats exposed to 300 ppm UG, whereas no increase in RCT was observed in any exposure group. It is concluded that UG and TMP are promoters of ACF and RCT in male, but not female, rats under the conditions of this study. Data from related investigations suggest that the tumor promoting potential of UG and TMP results from reversible binding of metabolites to α2u-globulin, which leads to decreased renal catabolism of this protein, chronic lysosomal overload, cell death, and compensatory cell proliferation.

INTRODUCTION

The multistage concept of carcinogenesis, first described for skin (1), has been extended to many organ systems, including liver, colon, pancreas, lung, urinary bladder, and kidney (2, 3). The simplest multistage model of carcinogenesis consists of two stages: an irreversible initiation phase, followed by a series of, at least initially, reversible, promotion steps that result in the formation of a neoplasm (4). Since most human cancers are believed to arise from environmental exposure and lifestyle (5), determination of the ability of a chemical carcinogen to initiate and/or promote tumor formation in experimental animals should improve our ability to extrapolate animal data to human risk. Several nephrotoxic chemicals have demonstrated carcinogenic potential in chronic bioassays (6). Unleaded gasoline has been shown to induce a syndrome known as α2u-globulin nephropathy that is associated with a low, but dose related, increase in renal adenomas and adenocarcinomas of male F344 rats (7, 8). Neither female rats nor mice of either sex developed α2u-G2 nephropathy or renal tumors when similarly exposed. Nonneoplastic lesions that develop in kidneys of male rats with this syndrome include the accumulation of protein droplets in the P2 segment of the proximal tubule, granular casts, tubular cell karyomegaly, hyperplasia of basophilic tubules, and increased severity of CPN (9, 10). In addition, exposure to UG for up to 1 year causes marked increases in cell proliferation to occur in the P2 and, to a lesser extent, P3 segment of the proximal tubule (10, 11).

Elucidation of the carcinogenic mechanism of UG is vital for determining the potential human health risk from exposure to this and other widely used chemicals that cause α2u-G nephropathy. UG may be a promoter of renal carcinogenesis, since it was shown to be nongenotoxic in most assays (12–15). Although many mechanisms for promotion exist, sustained increases in cell proliferation of renal tubules may prove to be an essential component of the carcinogenic response of many nongenotoxic nephrotoxins, including UG. Acute and chronic cytotoxic injury induced by UG and other nongenotoxic nephrotoxins have been associated with elevated replicative rates of tubular epithelial cells in a site specific manner (10, 11, 16–19). In addition, acceleration of CPN observed following light hydrocarbon exposure of male rats may contribute promoting potential (11, 20).

An initiation-promotion study of UG and nephrotoxic components of UG, such as TMP, would provide important information for elucidating the carcinogenic mechanism of UG. EHEN is a complete carcinogen which selectively causes renal epithelial tumors of the proximal renal tubule, as well as liver tumors in rats (21, 22). Many proximal tubular toxins have been shown to promote kidney tumors of rats following initiation with 500 to 1000 ppm EHEN in the drinking water for 1 to 2 weeks (22). The nephrotoxins β-cyclodextrin (23), basic lead acetate (24), folic acid (25), DL-serine (26), trisodium nitrilotriacetate monohydrate (27, 28), potassium bromate (29), aminophenol (30), and nickel chloride (31) have all been shown to promote or enhance renal tumorigenesis in rats initiated with EHEN. Polychlorinated biphenyl induced damage of the proximal tubular epithelium but did not enhance the induction of renal tumors (32).

Previous initiation-promotion studies of renal tumorigenesis have yielded minimal information on sex comparisons, dose-response relationships, reversibility of preneoplastic and neoplastic lesions, and possible cocarcinogenic effects of test compounds as investigated in skin and liver (33–35). Therefore, to determine the carcinogenic mechanism(s) of UG, an initiation-promotion experiment in the kidney of F344 rats exposed via inhalation to UG or TMP was implemented that included both sexes of rats, exposure over a wide range of UG concentrations, short term versus long term promotion, and investigations on the effect of reversing the sequence of exposure to EHEN and TMP results from reversible binding of metabolites to α2u-globulin, which leads to decreased renal catabolism of this protein, chronic lysosomal overload, cell death, and compensatory cell proliferation.

Received 12/14/88; revised 6/13/89; accepted 8/14/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Present address: Smith Kline and French Laboratories, P. O. Box 1539, King of Prussia, PA 19406-0399.

2 Current address: Dept. of Pathology, University of North Carolina, Chapel Hill, NC 27514. To whom requests for reprints should be addressed.

3 The abbreviations used are: α2u-G, α2u-globulin; UG, unleaded gasoline; CPN, chronic progressive nephropathy; TMP, 2,2,4-trimethylpentane; EHEN, N-ethyl-N-hydroxyethylnitrosamine; ACF, atypical cell foci; RCT, renal cell tumors; O6-EtdG, O'-ethyldeoxyguanosine.

6369
Chromatograph was calibrated by preparing a series of UG and TMP ber. carried the vaporized UG or TMP from the J-tube to the chamber for exposure periods that lasted 24 or 59 to 61 weeks. Test exposed 6 h/day, 5 days/week to 0, 10, 70, or 300 ppm UG or 50 ppm TMP for 24 or 59 to 61 weeks by inhalation. Ambient concentrations of UG were selected based on the predicted no observed effect level (10 ppm UG), on previous bioassay results of 1 of 60 and 4 of 60 renal cell tumors for 70 and 300 ppm UG, respectively (7, 8), and on their ability to increase renal cell proliferation (10). The 50-ppm TMP exposure concentration was chosen based on its predicted ability to induce proximal tubular cell proliferation (10, 17).

MATERIALS AND METHODS

Animals

Eight hundred forty male and 390 female F344 rats approximately 5 to 6 weeks of age were obtained from Charles River Breeding Laboratories (Raleigh, NC). Barrier sustained animal rooms were maintained at 22 ± 1°C with a 12-h light-dark cycle. Animals were allowed free access to food (NIH-07 rat chow; Ziegler Brothers, Gardners, PA) and tap water except during the actual exposures. Following a 3-week quarantine period, 740 male and 350 female rats were randomized according to body weight into groups (see below) and individually housed in hanging stainless steel cages.

Chemicals

EHEN (purity 99.8%; liquid at room temperature) was purchased from Shigematsu & Co., Ltd. (Osaka, Japan) and mixed thrice weekly in distilled water for a final concentration of 0.017%. Samples from mixing containers and drinking dispensers on each animal rack were submitted for analysis three times per week prior to and after administration to assure accurate and consistent target concentrations. EHEN water samples were analyzed by reverse phase high performance liquid chromatography. Samples were diluted 1:10 with high performance liquid chromatography grade water, and an aliquot was injected into the mobile phase, which consisted of 50% methanol-50% water. Separation was obtained with a Waters RCM-100 column followed by a CN cartridge column. EHEN was detected at an absorbance of 230 nm.

Animal Housing and Chamber Exposures

Animals were housed and exposed in 8-m³ stainless steel and glass whole body inhalation chambers (36), which were operated under dynamic conditions of approximately 2200 liters/min of high efficiency particulate air-charcoal filtered, dried, and rehumidified air and at slightly subatmospheric pressure (0.1 to 0.3 in of water). Animals were exposed 6 h/day, 5 days/week to 0, 10, 70, or 300 ppm UG or 50 ppm TMP for exposure periods that lasted 24 or 59 to 61 weeks. Test atmospheres were generated by metering liquid UG or TMP into a heated, stainless steel J-tube (37) containing 5-mm glass beads. A stream of nitrogen (10 to 30 liters/min) passing through the J-tube carried the vaporized UG or TMP from the J-tube to the chamber supply air duct for mixing, before introduction to the inhalation chamber.

Chamber atmospheres were analyzed three times per h with a Hewlett-Packard 5880 gas chromatograph equipped with an automatic stream selector valve, 0.5-m sample loop, flame ionization detector, and 5-ft-long, 1/8-in-o.d. stainless steel column packed with 1/0.5% OV-101 on 100/120 mesh Chromosorb GHP operating at 200°C. The gas chromatograph was calibrated by preparing a series of UG and TMP standards in sealed glass flasks of predetermined volume. UG concentrations were calculated by using an average molecular weight of 108 and a density value of 0.7350 g/ml. Chamber environmental parameters were maintained nominally at 22°C (20–24°C) and 50% relative hu-midity (40 to 60%) and were recorded hourly during exposure. Nominal concentrations were determined daily.

Initiation-Promotion Protocols

Two weeks prior to the start of the experiment, rats were randomized by weight and divided into groups of 30 to 40 animals; male and female rats weighed 200 to 260 and 135 to 160 g, respectively. All surviving rats were sacrificed 65 to 67 weeks (mean, 66 weeks) after the start of the experiments, except for nine animals/sex/concentration from control and promotion control groups, which were sacrificed at earlier intervals for cell proliferation studies (11). The groups were as described below.

Hydrocarbon Promotion Study (Fig. 1)

Control. Thirty-one male and 31 female rats were administered purified drinking water and housed in inhalation chambers throughout the 65- to 67-week study.

Promotion Control. Following a 6-week control period, 124 male and 124 female rats were exposed to inhalation concentrations of 10, 70, or 300 ppm UG or 50 ppm TMP (31 rats/sex/concentration) until sacrifice.

Initiation Control. Thirty rats/sex were administered 170 ppm EHEN in the drinking water for 2 weeks, followed by purified water until sacrifice.

Experimental Initiation-Promotion. One hundred twenty male and 120 female rats were given 170 ppm EHEN in the drinking water for 2 weeks, followed by a 4-week holding period and then inhalation exposure to 10, 70, or 300 ppm UG or 50 ppm TMP (30 rats/sex/concentration) until sacrifice.

Sequence Study (Fig. 2)

Experimental Initiation-Promotion. One hundred twenty male rats (30 rats/concentration) were administered 170 ppm EHEN in the drinking water for 2 weeks, followed by a 4-week holding period. Rats were then exposed to 10, 70, or 300 ppm UG, or 50 ppm TMP for 24
weeks and then held until 65 to 67 weeks after the start of the experiment.

Promotion Control. One hundred twenty male rats (30 rats/concentration) were exposed to 10, 70, or 300 ppm UG or 50 ppm TMP for 24 weeks and then held until sacrifice at 65 to 67 weeks.

Reverse Sequence Initiation Control. Thirty male rats were administered 170 ppm EHEN in the drinking water during weeks 28 to 30 of the study and were held until sacrifice at 65 to 67 weeks.

Cocarcinogenesis Control. One hundred twenty male rats (30 rats/concentration) were exposed to 10, 70, or 300 ppm UG or 50 ppm TMP for 24 weeks, followed by a 4-week holding period, and then administered 170 ppm EHEN in the drinking water for 2 weeks and sacrificed at 65 to 67 weeks.

Biological Observations

Animals were observed twice daily for signs of toxicity, behavioral changes, and deaths. Clinical observations and body weights were recorded weekly for the first 2 months and biweekly thereafter. Gross and microscopic examinations of tissues were performed on animals dying during the study and on those animals sacrificed at termination. During weeks 65 to 67, surviving animals were anesthetized with an i.p. injection of 1 to 1.5 ml sodium pentobarbital (Nembutal) and exsanguinated. A complete necropsy was conducted on all animals.

Light Microscopic Examination

The kidneys were removed, weighed, and fixed in 10% phosphate-buffered formalin solution. Both kidneys from each rat were cut into six serial transverse slices, each 3 mm thick. Sections from each block were stained with hematoxylin and eosin.

The numbers of RCT and ACF were quantified in each section. Each animal was assigned a score of 1 to 4+ based on the severity and extent of CPN.

Statistical Procedures

Body weight, kidney weight, and arc sine transformation of relative kidney weight were tested for homogeneity of variance (38). Dunnett's test was conducted to determine significant differences ($P \leq 0.05$) from the control group. These and all other subsequent statistical tests were performed using an RS/1 computerized software package (BBN Software, Cambridge, MA). Results of CPN scores were analyzed by a Kruskal-Wallis nonparametric analysis of variance, with a suitable multicomparison test for significant ($P < 0.05$) differences from control. The incidence of ACF and RCT in the initiation-UG promotion (24 and 60 weeks) and initiation control groups was tested for significant trend by a Cochran-Armitage test. The incidence of ACF and RCT in the initiation-50 ppm TMP promotion (24 and 60 weeks) group was tested for significance against the initiation control group by a one-tailed Fisher's exact test. The total number of ACF and RCT of initiated and promoted animals was analyzed for significant increases compared to initiated controls by an analysis of variance and Dunnett's multicomparison test.

RESULTS

Chamber Conditions

Actual chamber concentrations during the 60- or 24-week promotion period or 24-week promotion reversal study averaged 10, 69, and 298 ppm UG and 50 ppm TMP, which were very close to target concentrations of 10, 70, or 300 ppm UG, or 50 ppm TMP, respectively.

General Animal Observations

Occasional clinical observations were noted during the study, including minimal ocular discharge, ruffled fur, and rare cases of alopecia over the ventral abdominal area in female rats. These changes were observed with similar frequency in control as well as in UG or TMP exposure groups. No significant differences were observed in spontaneous death rates of male or female rats, which did not exceed 12% in any group, including controls. Causes of spontaneous death or morbidity consisted of diseases commonly encountered in F344 rats which did not appear to be treatment related; including monoclonal cell leukemia, pituitary adenoma, mammary cancer, and a Zymbal's gland tumor. Several gliomas, hepatocellular carcinomas, and nasal adenocarcinomas were observed in animals given EHEN, but their incidence was low.

No significant terminal body weight differences were observed in any groups. Significant but mild depressions in kidney weight, 5 to 7% less than controls, were observed in 24-week promotion control male rats at each concentration of UG and TMP, 64-week promotion control males exposed to 10 ppm UG, and 50-ppm TMP promotion/initiation reversal males. Relative kidney/body weights in these groups of males were also significantly decreased by a similar margin. Kidney weights of several groups of female rats exhibited mild 5 to 7% increases compared to controls, but relative kidney/body weights were unaffected.

Pathologic Findings

Gross Observations

Gross examination revealed four renal cell tumors in male rats, with none found in females. All tumors were less than 3 mm in diameter, and they projected from the capsular surface. White focal areas and masses in the liver were observed in all groups of male and female rats exposed to EHEN. No intergroup differences were present based on gross examination (data not shown).

Morphology of Renal Lesions

Microscopic observations of the kidney are summarized for the hydrocarbon promotion study of male and female rats and the sequence study in male rats in Tables 1 to 3, respectively. CPN lesions were characterized as tubules lined by a single epithelial layer of cells with scant, basophilic cytoplasm that did not appear to be treatment related; including mononuclear cell leukemia, pituitary adenoma, mammary cancer, and a Zymbal's gland tumor. Several gliomas, hepatocellular carcinomas, and nasal adenocarcinomas were observed in animals given EHEN, but their incidence was low. No significant terminal body weight differences were observed in any groups. Significant but mild depressions in kidney weight, 5 to 7% less than controls, were observed in 24-week promotion control male rats at each concentration of UG and TMP, 64-week promotion control males exposed to 10 ppm UG, and 50-ppm TMP promotion/initiation reversal males. Relative kidney/body weights in these groups of males were also significantly decreased by a similar margin. Kidney weights of several groups of female rats exhibited mild 5 to 7% increases compared to controls, but relative kidney/body weights were unaffected.

<p>| Table 1 | Atypical cell foci and renal cell tumors in UG and TMP initiation-promotion study in male rats sacrificed at 65 weeks |</p>
<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>n</th>
<th>CPN* score</th>
<th>Rats affected (%)</th>
<th>No.</th>
<th>Rats affected (%)</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>1.4 ± 0.1*</td>
<td>1 (3.6)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Promotion control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 UG</td>
<td>27</td>
<td>1.2 ± 0.1</td>
<td>2 (7.4)</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>70 UG</td>
<td>30</td>
<td>1.4 ± 0.1</td>
<td>2 (6.7)</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>300 UG</td>
<td>30</td>
<td>1.6 ± 0.1</td>
<td>4 (13.3)</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50 TMP</td>
<td>28</td>
<td>1.6 ± 0.1</td>
<td>5 (17.9)</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Initiation control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHEN</td>
<td>29</td>
<td>1.4 ± 0.1</td>
<td>10 (34.5)</td>
<td>12</td>
<td>1</td>
<td>3.5</td>
</tr>
<tr>
<td>Initiation/promotion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHEN/10 UG</td>
<td>28</td>
<td>1.2 ± 0.1</td>
<td>13 (46.4)</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EHEN/70 UG</td>
<td>27</td>
<td>1.4 ± 0.1</td>
<td>17 (63.0)</td>
<td>1</td>
<td>26</td>
<td>(3.7)</td>
</tr>
<tr>
<td>EHEN/300 UG</td>
<td>27</td>
<td>1.9 ± 0.1*</td>
<td>21 (77.8)</td>
<td>54*</td>
<td>2</td>
<td>7.4</td>
</tr>
<tr>
<td>EHEN/50 TMP</td>
<td>29</td>
<td>1.9 ± 0.1*</td>
<td>23 (79.3)</td>
<td>57*</td>
<td>4</td>
<td>13.8</td>
</tr>
</tbody>
</table>

* Based on 1 to 4+ score of minimal to severe chronic progressive nephropathy.
* Means ± SEM.
* Significantly greater than control, Kruskal-Wallis multicomparison test ($P < 0.001$).
* Significantly greater than control, Fisher's exact test ($P < 0.001$).
was surrounded by a thick basement membrane, increased numbers of chronic inflammatory cells, and fibrocytes. Adjacent glomeruli had segmental or diffuse sclerosis. Kidneys from each animal were assigned a score from 1 to 4, based on the number of glomeruli having segmental or diffuse sclerosis. Kidneys from each animal in the EHEN initiation-reversal control group (Tables 1 and 3). An apparent trend of increased CPN score in male rat kidneys was noted in other promotion-control and promotion-initiation reversal groups (Table 1) and in the 24-week initiation-promotion groups (Table 3). An apparent trend of increased CPN score in male rat kidneys was noted in other promotion-control and promotion-initiation reversal groups (Table 1) and in the 24-week initiation-promotion study sacrificed at 65 weeks (Fig. 1). Promotion-control (Fig. 2).

Table 2. Atypical cell foci and renal cell tumors in female rats from UG and TMP initiation-promotion study sacrificed at 65 weeks

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Atypical cell foci</th>
<th>Renal cell tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPN score</td>
<td>Rats affected (%)</td>
</tr>
<tr>
<td>Control</td>
<td>1.2 ± 0.1</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>Promotion control</td>
<td>10 UG</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>70 UG</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>300 UG</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>50 TMP</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Initiation control</td>
<td>EHEN/50</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Initiation/promotion</td>
<td>EHEN/10 UG</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>EHEN/70 UG</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>EHEN/300 UG</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>EHEN/50 TMP</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

* Based on 1 to 4+ score of minimal to severe chronic progressive nephrosis.

Table 3. Atypical cell foci and renal cell tumors in sequence study of male rats promoted for 24 weeks with UG or TMP and sacrificed at 65 weeks

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Atypical cell foci</th>
<th>Renal cell tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPN score</td>
<td>Rats affected (%)</td>
</tr>
<tr>
<td>Control (Fig. 1)</td>
<td>1.4 ± 0.1</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>Promotion control (Fig. 2)</td>
<td>10 UG/control</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>70 UG/control</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>300 UG/control</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>50 TMP/control</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Initiation control (Fig. 1)</td>
<td>EHEN</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Initiation/promotion (Fig. 2)</td>
<td>EHEN/10 UG/control</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>EHEN/70 UG/control</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>EHEN/300 UG/control</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>EHEN/50 TMP/control</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Initiation control (wk 30, Fig. 2)</td>
<td>EHEN</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Cocarcinogenesis control (Fig. 2)</td>
<td>10 UG/EHEN/control</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>70 UG/EHEN/control</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>300 UG/EHEN/control</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>50 TMP/EHEN/control</td>
<td>2.2 ± 0.1</td>
</tr>
</tbody>
</table>

* Based on 1 to 4+ score of minimal to severe chronic progressive nephrosis.

Mean ± SEM.

UNLEADED GASOLINE RENAL CARCINOGENESIS IN RATS

Concentrations of UG or TMP exposure were mild karyomegaly of epithelial cells and mild to moderate mineralization of the papilla of male rats. However, the inconsistent appearance of karyomegaly changes and the relative insignificance of papillary mineralization to tumor formation did not warrant their analysis for treatment effect.

Histological changes indicative of preneoplastic or neoplastic lesions were defined as follows: ACF were composed of one (Fig. 3A) or several solid (Fig. 3B) or dilated (Fig. 3C) tubes containing slightly enlarged or multilayered tubular epithelial cells with generally enlarged nuclei containing vesicular, prominent nucleoli. The cytoplasm was pale staining or basophilic. These lesions were similar in description and appearance to ACF described elsewhere (26, 28, 39, 40) and were considered synonymous with such terms as dysplastic foci (30, 32), basophilic tubules (41–43), or "dysplastic tubular epithelium" (44), previously described in rats given EHEN or other closely related nitrosamines.

RCT were composed of nests of proliferated epithelial cells with pale staining, large, vesicular nuclei. Tumors were often solid, forming single or multiple nodules (Fig. 4, A and B), but occasional cystic forms had widened luminal spaces lined by several layers of proliferating cells, with or without projection into the lumen. Some of the larger tumors contained a central area of necrosis. Occasional small RCT with solid or cystic growth patterns were surrounded by tubules involved in CPN (Fig. 4C). The cytoplasm of RCT was characteristically basophilic, whereas granular eosinophilic (oncocytic) and clear cell (chromophobic) tumors were rarely observed. A total of 397 ACF and 28 RCT were observed microscopically in the entire study. Four of the tumors were noted macroscopically, and all were 3 mm or less in diameter. There were no apparent intergroup or sex related differences in the morphology of ACF or RCT. Metastasis of RCT was not observed, and the neoplasms were considered adenomas.
Fig. 3. ACF observed in kidney cortex of animals initiated with EHEN. A. small focus comprised of one enlarged tubule filled with hypertrophic cells containing vesicular nuclei. B. ACF composed of several enlarged tubules. C. cystic dilation of ACF containing necrotic cell debris. H & E stain. Bar = 22 μm (A), 30 μm (B, C).
Fig. 4. RCT observed in kidney cortex of animals initiated with EHEN. A, low power magnification of nodule in cortex projecting through capsular surface. B, higher magnification of A; tumor composed of solid nests of epithelial cells invading adjacent parenchyma. C, small solid RCT within focus of CPN characterized by thickened basement membranes around tumor and adjacent renal tubules, renal interstitial infiltrate, and sclerotic glomerulus. H & E stain. Bar = 300 μm (A); 22 μm (B); 30 μm (C).
Incidence and Total Number of ACF and RCT

Hydrocarbon Promotion Study. Male Rats (Table 1). There was an increased linear trend in incidence of animals with ACF (34 to 78%) from groups exposed to EHEN followed by 0 to 300 ppm UG. Similarly, the group exposed to 50 ppm TMP following EHEN had a greater ACF incidence (79%) than EHEN controls (34%). In addition, the total numbers of ACF in groups exposed to EHEN followed by 300 ppm UG (54 ACF) or 50 ppm TMP (57 ACF) were significantly greater than the EHEN control group (12 ACF). Both the incidence and total number of RCT within groups of males exposed to UG or TMP and initiated with EHEN were elevated over the initiated control group; however, the increase did not achieve statistical significance.

Female Rats (Table 2). No significant elevations in incidence or total numbers of ACF or RCT were observed in any experimental group compared to appropriate controls.

Sequence Study (Table 3). The total number of ACF for animals initiated and promoted for 24 weeks was roughly one-half of that observed in animals promoted for 60 weeks at the respective concentrations. There was a significant trend in ACF incidence (24 to 50%) of animals exposed to EHEN followed by exposure to 24 weeks of 0 to 300 ppm UG. The initiation and 50-ppm TMP promotion group in this experiment also exhibited an increased ACF incidence (60%) compared to controls (34%). There was a significant linear trend in RCT of groups exposed to UG for 24 weeks following EHEN. The maximal tumor incidence was similar to that observed with the longer promotion period. The RCT incidence of the group promoted for 24 weeks with 50 ppm TMP (13%) was similar to the maximal response observed in the UG groups (14%). The increase in TMP promoted RCT was not statistically significant, probably due to the use of the Cochran-Armitage test for the UG concentration groups and Fisher’s exact test for TMP.

In the reversal study, the group exposed to 300 ppm UG for 24 weeks and then treated with EHEN at week 30 had a slightly higher incidence of ACF compared to the initiation reversal control group. No other effects on RCT or ACF in other concentration groups were observed in this experiment.

DISCUSSION

The hydrocarbon promotion and sequence reversal studies reported here demonstrate that UG and TMP are promoters of ACF and renal adenomas in male, but not female, rats. These conclusions have been drawn after evaluation of the data on biological and statistical grounds. Statistical analysis (Cochran-Armitage trend test) of the incidence of RCTs during 24 or 60 weeks of UG promotion demonstrated a promoting effect at 24 (P < 0.02) but not 64 weeks (P < 0.10). Since the total number of renal adenomas induced in these studies was small, additional insight can be gained by summation of the tumors from the two promotion regimens. Indeed, the P value of this summation (P < 0.01) further substantiates the promoting effect of UG on RCT. Exposure to 50 ppm TMP resulted in similar numbers of RCT in male, but not female, rats initiated with EHEN. The number of RCT was not statistically significant, even though there was a higher tumor incidence in TMP promoted groups (8 of 59 = 14%) than in 300-ppm UG promoted groups (6 of 55 = 11%) due to the use of Fisher’s exact test.

The low number of renal neoplasms in the study probably reflects a lower level of renal initiation, compared to previous studies (22). A 10% incidence of renal tumors in initiated control animals may have been more desirable for demonstrating statistically significant increases in RCT of promoted animals. The concentration of EHEN given in the drinking water, the age of animals at the time of EHEN administration, and the amounts of DNA alkylation are thought to be critical determinants of the extent of initiation (18). Previous investigators have utilized 500 ppm EHEN for 2 weeks as an initiating dose for two-stage kidney carcinogenesis experiments in male rats (29, 31, 44). The reported renal tumor incidences, which ranged from 13 to 20% in 26-week studies, prompted us to decrease the concentration of EHEN in our 65- to 67-week studies to avoid an undesirably high incidence of RCT in initiated controls. In addition, the rats used in this study were 10 to 11 weeks old during initiation, which was 3 to 4 weeks older than rats used in the previous studies. The kidneys of these slightly older rats may not have been undergoing the same rate of cell proliferation compared to younger rats. The extent of cell proliferation has been shown to be a critical factor in initiation (45). Alternatively, metabolic activation of EHEN may be influenced by age, as observed with other renal carcinogens (46). A study was conducted in male F344 rats to determine the concentration of the promutagenic DNA adduct O6-EtdG after consummation of 170 to 500 ppm EHEN in the drinking water for 2 weeks. The surprising result was that lowering the 500 ppm EHEN dose to 170 ppm (33% of 500 ppm) lowered the O6-EtdG levels from 1.7 to 10^-7 to 2.0 x 10^-8 mol O6-EtdG per mol guanine, respectively.

J. Boucheron, B. G. Short, and J. A. Swenberg, unpublished data.
segment of male rats persisted for up to 10 days after termination of 10 or 22 weeks of exposure to 300 ppm UG or 50 ppm TMP. If similar increases in cell proliferation continued during administration of EHEN, they would be expected to increase "fixation" of EHEN-induced promutagenic DNA damage in renal cells. This interpretation is further supported by the presence of increased numbers of regenerative tubules and other lesions associated with α₂uG nephropathy 4 weeks after exposure to Stoddard solvent or C₁₀H₂₃C₁₇ isoparaffins (53) or unleaded gasoline (54). In addition, the acceleration of CPN, as seen in all groups of animals exposed to 300 ppm UG or 50 ppm TMP, may have contributed to the increased ACF incidence via increased cell turnover of renal tubules involved in CPN (11).

The precise anatomical origin of the RCT and ACF arising in this initiation-promotion study cannot be determined with certainty. However, previous investigations with EHEN and other chemical carcinogens have demonstrated that neoplastic and neoplastic lesions induced in the rat kidney are of proximal tubular histogenesis, based on ultrastructural (55–57) and histochemical examination (49, 50, 58). The origin of oncocytoma in man and animals has been shown to be the collecting ducts (40, 59). However, oncocytomas were rare in this study and were insignificant compared to the number of basophilic RCT. The specific proximal tubular segment of origin of basophilic tumors induced by EHEN is unknown, but the majority of the ACF and RCT in this study were observed in the cortex, where P₁, P₂, and early P₃ segments reside. Fewer ACF and RCT were noted in the outer stripe of the outer medulla, where most P₃ segments are located. Reports of histogenesis with other carcinogenic nitrosamines indicate that the proximal tubular segment origin varies with the chemical. For instance, diethylnitrosamine induces tumors of the P₂ and cortical P₃ segments (40), whereas dimethylnitrosamine induced tumors are believed to arise from the initial (P₁) segment of the proximal convoluted tubule (41).

The sex and species specificity of the nephrocarcinogenicity of UG is intriguing, and it has stimulated much research on the biochemical and pathological effects of UG and TMP in male versus female rats (60). Recent studies conducted in this laboratory suggest that the promoting activity of UG and TMP in male rats is due to chronically elevated cell proliferation of proximal tubules (11). The P₂ segment of the proximal tubule of male, but not female, rats had 4- to 11-fold increases in cell proliferation during up to 48 weeks of inhalation exposure to 300 ppm UG and 50 ppm TMP. Excessive cell proliferation of this segment was induced by lysosomal accumulation of the male rat specific protein, α₂uG, which caused recurrent cytotoxicity and restorative hyperplasia within this segment of the nephron. α₂uG, a low molecular weight protein of postpubertal male rats, is synthesized by the liver under multihormonal control. This protein is reabsorbed by the proximal tubule, but exposure of rats to UG, TMP, and other environmental chemicals induces a protein droplet nephropathy, characterized by acute and chronic cytotoxicity and regenerative hyperplasia of cells of the P₂ segment, which is the primary proximal tubule segment involved in accumulating the protein (10, 11, 16, 17, 60–62). Studies with TMP in male rats have demonstrated reversible binding between α₂uG and a metabolite of TMP, as well as decreased digestibility of this complex compared to native α₂uG (60). The accumulated α₂uG induces a protein droplet nephropathy that has not been observed in female rats or in mice, dogs, or monkeys of either sex (53, 63). The 6 to 25% incidence of RCT observed at maximum tolerated doses in chronic bioassays of chemicals causing α₂uG nephropathy...
UNLEADED GASOLINE RENAL CARCINOGENESIS IN RATS


23. Williams, G. M., and Furuya, K. Distinction between liver neoplasia promoting and synergistic carcinogenic effects demonstrated by exposure to phenobarbital or diethylnitrosamine either before or after N2-fluorenylacetamide. Carcinogenesis (Lond.), 5: 611-616, 1984.


Promoting Effects of Unleaded Gasoline and 2,2,4-Trimethylpentane on the Development of Atypical Cell Foci and Renal Tubular Cell Tumors in Rats Exposed to N-Ethyl-N-hydroxyethylnitrosamine

Brian G. Short, William H. Steinhagen and James A. Swenberg


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/22/6369