

# Differential Effects of CYP2E1 Status on the Metabolic Activation of the Colon Carcinogens Azoxymethane and Methylazoxymethanol<sup>1</sup>

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## ABSTRACT

Methylazoxymethanol (MAM) and its chemical and metabolic precursor, azoxymethane (AOM), both strong colon carcinogens in rodents, can be metabolically activated by CYP2E1 *in vitro*. Using CYP2E1-null mice, we found that CYP2E1 deficiency differentially affects the activation of AOM and MAM, as reflected in DNA guanine alkylation in the colon and in the formation of colonic aberrant crypt foci (ACF). Male and female inbred 129/SV wild-type (WT) and CYP2E1-null (null) mice were treated with 189  $\mu\text{mol/kg}$  of either AOM or methylazoxymethyl acetate (MAMAc), and 7-methylguanine (7-MeG) and *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-MeG) were measured in the DNAs of various organs. The levels of *O*<sup>6</sup>-MeG (as pmol/nmol guanine) in the liver, colon, kidney, and lung of male null mice treated with AOM were 87, 48, 70, and 43% lower, respectively, than in AOM-treated WT mice. In null mice treated with MAMAc, the DNA *O*<sup>6</sup>-MeG levels were lower by 38% in the liver but were higher by 368, 146, and 194% in the colon, kidney, and lung, respectively, compared with the same organs of WT mice treated in the same way. Determination of ACF revealed that although AOM-induced ACF formation was significantly lower in the null group than in the WT group, MAMAc-induced ACF formation was significantly higher in the null group than in the WT group. These results demonstrate an important role for CYP2E1 in the *in vivo* activation of AOM and MAM and suggest that agents that modify CYP2E1 activity at the tumor initiation stage might either enhance or inhibit colon carcinogenesis, depending on whether AOM or MAMAc is used as the carcinogen. The mechanism of this effect is discussed.

## INTRODUCTION

MAM,<sup>3</sup> MAMAc, the stable acetic acid ester of MAM, and AOM, the chemical and metabolic precursor of MAM, are potent colon carcinogens in rodents (1–4). These compounds, especially AOM, have been used in numerous studies seeking to identify and evaluate potential colon cancer chemopreventive agents. In those cases where the agent modifies colon carcinogenesis at the stage of tumor initiation, it is important to determine whether carcinogen metabolism is being influenced and, if so, to identify the enzymes involved. This information seems a prerequisite to evaluating the relevance of the results to humans. We carried out the present work to determine the role of cytochromes P-450, specifically CYP2E1, in the metabolic activation of AOM and MAM to a DNA-alkylating species and in the development of colonic ACF. The latter represent precursor lesions of colon cancer (5–7).

As shown in Fig. 1, AOM is converted to MAM by hydroxylation of the methyl group distal to the N(O) function. This reaction, which was predicted by Druckrey (1), was subsequently shown to be cata-

lyzed by CYP2E1 *in vitro* (8). Colon cancer chemopreventive agents such as disulfiram, now known to be an inhibitor of CYP2E1 (9), blocked the metabolism of <sup>14</sup>C-labeled AOM *in vivo* (10), providing evidence that the reaction also occurred *in vivo*. MAM is an unstable compound with a half-life of about 12 h under physiological conditions (11, 12), spontaneously decomposing to formaldehyde and a highly reactive alkylating species, probably the methyldiazonium ion (11). The latter is responsible for the alkylation of DNA guanine to 7-MeG and *O*<sup>6</sup>-MeG and of thymine to *O*<sup>4</sup>-methylthymine (13–16). *O*<sup>6</sup>-MeG and *O*<sup>4</sup>-methylthymine are miscoding base modifications, which, if not repaired prior to DNA replication, can represent initiation of tumorigenesis.

It was first proposed by Schoental (17) that MAM might be enzymatically oxidized to methylazoxyformaldehyde, specifically in a reaction catalyzed by ADH, a cytosolic enzyme. This proposal was attractive because a tissue-specific enzymatic activation step, rather than spontaneous decomposition, seemed necessary to explain the narrow range of organs in which MAM induces tumors—the colon, liver, and kidney. In mice and rats, ADH activity is present in all three of these organs (18). The ability of ADH to metabolize MAM to a reactive methylating species *in vitro* was demonstrated by Zedeck and co-workers (12, 19). However, to show unequivocally that ADH was also responsible for the activation of MAM *in vivo* by pretreatment of rats with ADH inhibitors such as pyrazole (19) was difficult, because pyrazole inhibits not only ADH but also microsomal mixed function oxidase activity (20). To determine whether ADH is necessary for the *in vivo* metabolic activation of MAM, the metabolism of <sup>14</sup>C-labeled MAMAc was determined in two strains of the deer mouse (*Peromyscus maniculatus*), one of which has a normal complement of class I ADH and the other, which is deficient in the enzyme and has a greatly reduced ability to metabolize ethanol (21). Both strains, however, contain an ethanol-inducible, microsomal ethanol-metabolizing system, which is elevated ~2-fold in the ADH-negative strain. Because the rates of metabolism of MAM were the same in both mouse strains, as were the levels of liver DNA guanine alkylation, these studies concluded that although it was possible that ADH was involved in MAM metabolism, its involvement was not stringent—clearly, MAM was metabolized by enzymes other than ADH in the ADH-negative deer mice (22). This conclusion was further supported by the finding that washed liver microsomes obtained from either deer mouse strain could metabolize MAM in the presence of NADPH; moreover, the rate of MAM metabolism by the microsomes from the ADH-negative mice was about twice that obtained with microsomes from the ADH-positive mice (22). Later studies showed that purified CYP2E1 could catalyze the oxidation of MAM as well as of AOM, and that a specific monoclonal antibody toward CYP2E1 inhibited these reactions (8). Finally, the participation of CYP2E1 in the metabolism of AOM as well as of MAM *in vivo* is shown unambiguously in the present work, which uses CYP2E1-null and wild-type mice. Importantly, our results suggest the apparent paradox that colon carcinogenesis may be either inhibited or enhanced by putative chemopreventive agents that are administered during the initiation stage, depending on whether the agents inhibit or induce CYP2E1 and on whether AOM or MAMAc is used as the carcinogen.

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<sup>3</sup> The abbreviations used are: MAM, methylazoxymethanol; MAMAc, methylazoxymethyl acetate; WT, wild-type; null, CYP2E1-null mice; AOM, azoxymethane; ADH, alcohol dehydrogenase; 7-MeG, 7-methylguanine; *O*<sup>6</sup>-MeG, *O*<sup>6</sup>-methylguanine; ACF, aberrant crypt foci; NDMA, *N*-nitrosodimethylamine; *p*-NP, *p*-nitrophenol; 4-NC, 4-nitrocatechol; HPLC, high performance liquid chromatography.

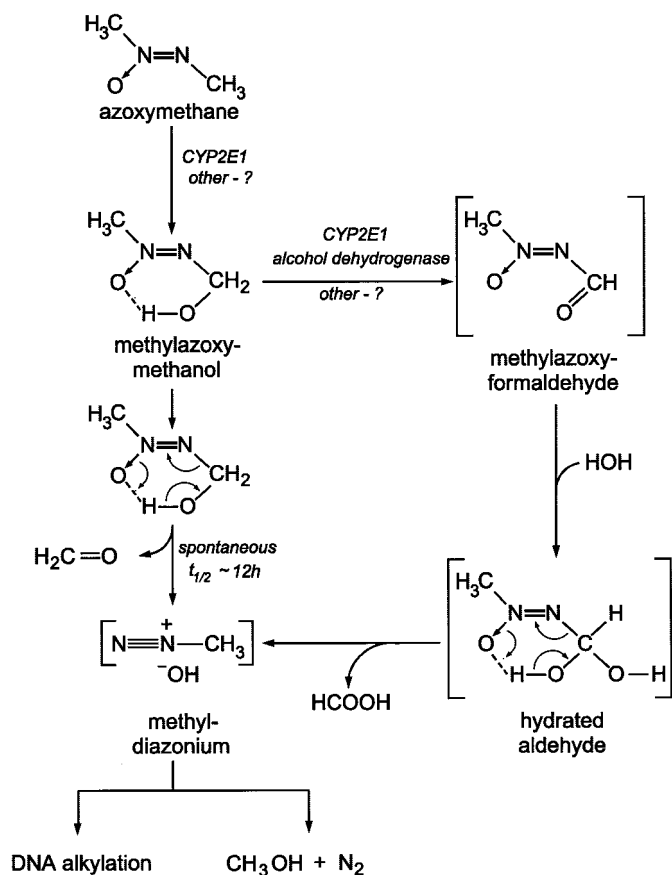


Fig. 1. Current views on metabolic pathways of AOM and MAM activation (1, 2, 11, 12, 54). AOM is hydroxylated to MAM by CYP2E1, and perhaps by other P450 isoenzymes, in the liver. MAM can be oxidized in reactions catalyzed by CYP2E1 and/or ADH in the liver or by ADH or other enzymes in extrahepatic organs. The oxidized product, methylazoxyformaldehyde, is envisioned to add water and, with the release of formic acid, to yield the methyl-diazonium ion, which methylates DNA bases in AOM and MAM target organs. MAM can also spontaneously decay ( $t_{1/2}$ ,  $\sim 12$  h) to the methyl-diazonium ion with the release of formaldehyde. Because treatment with either AOM or MAMAc results in detectable levels of DNA alkylation even in CYP2E1-null mice (see text and Table 2), it is apparent that the hydroxylation of AOM and the oxidation of MAM also involve enzymes other than CYP2E1.

## MATERIALS AND METHODS

**Chemicals.** The sources of the chemicals used were as follows. AOM was from Ash Stevens, Inc. (Detroit, MI); MAMAc, NDMA, 4-NC, and coumarin were from Aldrich Chemical Co. (Milwaukee, WI). *p*-NP, NADP<sup>+</sup>, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, erythromycin, ethoxyresorufin, and pentoxyresorufin were from Sigma Chemical Co. (St. Louis, MO). Methoxyresorufin was from Molecular Probes (Eugene, OR).

**Animals.** All animal experiments in these studies were conducted under federal guidelines for the use and care of laboratory animals and approved by the American Health Foundation Institutional Animal Care and Use Committee. One male and 2 female CYP2E1-null mice (*cyp2e1*<sup>-/-</sup>, null) and 2 male and 4 female of wild-type (*cyp2e1*<sup>+/+</sup>, WT) counterparts were obtained from the National Cancer Institute and were bred and reared at the American Health Foundation Research Animal Facility. The breeder animals were screened for viral infections prior to receipt at the Facility; all tests were negative. Animals were maintained in the animal-holding room under controlled environmental conditions (12/12 h light/dark cycle, 50% humidity, and 21°C) and fed NIH-07 rodent chow pellets. The animals were  $\sim 8$  weeks of age at the beginning of each study described below.

**Enzyme Assays.** To confirm the CYP2E1 status of null mice and to determine whether other enzyme activities differed between the WT and null mice, various substrate-specific assays were conducted using microsomes and/or cytosols prepared from the livers and colons. Male and female mice of each type, 3 animals/group, were used in this experiment. Cytosols and

microsomal fractions of liver and colon mucosa were prepared as described previously (23). Protein was determined by the method of Lowry *et al.* (24). In all assays, spectral determinations were made using a Cintra 40 UV-visible spectrometer (GBC Scientific Equipment Pty. Ltd., Dandenong, Victoria, Australia) or a Perkin-Elmer 650-10S fluorescence spectrophotometer. ADH was assayed in the cytosol fractions of the livers and of colon mucosae by the reverse reaction (25) in an assay mixture consisting of 0.14 mM NADH, 2 mM acetaldehyde, and 0.2–0.8 mg cytosolic protein in 1.2 ml of 3.3 mM sodium PP<sub>i</sub>, pH 6.5. The oxidation of NADH to NAD<sup>+</sup> was monitored by the decrease in absorbance at 340 nm. Activities of CYP2E1 in liver microsomes were determined by monitoring NDMA demethylation and *p*-NP hydroxylation essentially as described (26, 27). The incubation system for NDMA demethylation consisted of an NADPH-generating system (3.5 mM glucose 6-phosphate, 1.5 mM NADP<sup>+</sup>, 5 units of glucose-6-phosphate dehydrogenase, and 3.5 mM MgCl<sub>2</sub>), 1 mM NDMA, and 1.0 mg microsomal protein in 1.0 ml 0.1 M Tris buffer (pH 7.0). After incubating the mixture for 15 min, the amount of formaldehyde produced from the enzymatic demethylation was assayed as described previously (23). The incubation system for *p*-NP hydroxylation consisted of the NADPH-generating system, 0.2 mM *p*-NP, and 1.0 mg of microsomal protein in 1.0 ml of 0.05 M Tris buffer (pH 7.4). After 10 min, reactions were terminated by the addition of 0.25 ml of 0.6 N perchloric acid. After centrifugation, 1.0 ml of the supernatant was mixed with 50  $\mu$ l of 10 N NaOH, and absorbance was read at 512 nm (27). Enzyme activities were calculated from a 4-NC standard curve, and the incubation mixture without substrate was used as the blank. For other cytochrome P-450 isozymes, 7-alkoxyresorufin dealkylase activities were used to determine CYP1A1, CYP1A2, or CYP2B1 levels using ethoxyresorufin (28), methoxyresorufin (29), or pentoxyresorufin (30) as substrate, respectively. Erythromycin demethylase (31) and coumarin 7-hydroxylase (32, 33) were used to determine CYP3A4 and CYP2A5 levels, respectively, with some modifications described previously (23).

**Assay of DNA Guanine Alkylation in Mouse Organs.** Male or female WT or null mice, 6 per group, received s.c. injections with 189  $\mu$ mol/kg of either AOM (14 mg/kg) or MAMAc (25 mg/kg) dissolved in 0.9% NaCl. Because of the greater chemical stability of MAMAc compared with MAM, MAMAc is the preferred agent in animal experiments, but the biological effects of the two compounds are equivalent, because the blood and various tissues contain deacetylases, which rapidly hydrolyze the ester to the alcohol (34). The animals were sacrificed 6 h later, and the livers, colons (slit open), lungs, and kidneys were rinsed with ice-cold 0.15 M NaCl/0.015 M sodium citrate buffer (pH 7.0) and stored at  $-70^{\circ}\text{C}$ . DNA from these organs was isolated using the Boehringer Mannheim DNA Isolation kits for cells and tissues (purchased from Roche Molecular Systems). However, to eliminate contamination of the DNA with RNA (as determined by HPLC analysis of nucleosides after enzymatic hydrolysis), we found it was necessary to use amounts of RNase in excess of those specified in the manufacturer's original procedure. For liver samples,  $\sim 200$  mg of the tissue were homogenized in 5 ml of cellular lysis buffer and then incubated for 1 h at  $37^{\circ}\text{C}$  in the presence of 3.1  $\mu$ l of proteinase K solution. After this incubation, 200  $\mu$ l of the RNase solution provided by the manufacturer was added, together with an additional 200  $\mu$ l of RNase A solution (type II-A, bovine pancreas, 10 mg/ml) and 200  $\mu$ l of RNase T<sub>1</sub> solution (*Asperigillus oryzae*, 10,000 unit/ml), both prepared in 10 mM Tris/15 mM NaCl (pH 7.4), and heated at  $100^{\circ}\text{C}$  for 15 min to inactivate DNase. The samples were then further incubated 15 min at  $37^{\circ}\text{C}$ . After the incubation, 2.1 ml of the manufacturer's "protein precipitation solution" was added, and the sample was centrifuged at  $26,900 \times g$  for 20 min at room temperature. The supernatant was mixed with 5 ml of isopropanol, and the DNA strings were washed three to four times with 70% ethanol. The DNA was dissolved in 250  $\mu$ l of 10 mM Tris (pH 7.0) and stored at  $-20^{\circ}\text{C}$  until analysis for methylated guanines. For the colon mucosae, lungs, and kidneys, 75–100 mg of tissue were used for the DNA isolation; correspondingly, the amount of each reagent used was one-half of that used for the liver samples. DNA purified in this way was hydrolyzed in 0.1 N HCl at  $37^{\circ}\text{C}$  for 18 h. After determination of absorbance at 266 nm, portions of hydrolyzates were submitted to HPLC in a method adapted from Herron and Shank (15, 35). The HPLC system consisted of a Shimadzu HPLC pump, an SSI 500 variable UV/Vis detector, a Hewlett Packard (HP) Series 1100 fluorescence detector, and an HP 35900E Interface. The Waters Millennium software was used for data acquisition and analysis. The HPLC column system, consisting of a

Whatman Partisil SCX column (0.46 × 25 cm, 10 μm) with a Brownlee Aquapore CX-300 guard column (Perkin-Elmer, Norwalk CT) was eluted with 50 mM (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> buffer (pH 2.5) at a rate of 2 ml/min. The effluent was sequentially monitored for absorbance at 276 nm for quantifying guanine and for fluorescence at 370 nm (excitation, 295 nm) for quantifying O<sup>6</sup>-MeG and 7-MeG.

**AOM- and MAMAc-induced Colon ACF Formation.** Male and female WT or null mice, 10 mice/group, were treated with a single s.c. injection of either AOM (10 mg/kg), MAMAc (9 mg/kg), or 0.9% NaCl. All mice were killed 3 weeks after treatment. The colons were removed, flushed with Krebs-Ringer solution, slit open from the cecum to the anus, and fixed flat between two pieces of filter paper in 10% buffered formalin. After a minimum of 24 h in formalin, the colons were placed in a solution of 0.2% methylene blue dissolved in Krebs-Ringer solution for 15–30 min, placed on a glass slide with the mucosa side up, and viewed with a light microscope at ×40. The colons were assessed for ACF as described by McLellan *et al.* (5).

**Statistical Analysis.** Group means were compared among the groups using one-way ANOVA followed by Fisher's protected *t* test.

## RESULTS

**Enzyme Assays.** The specific activities of CYP2E1 and other cytochrome P-450 isozymes in the livers of male and female CYP2E1-null and WT mice, as determined by substrate-specific enzyme assays, are shown in Table 1. Because ADH is likely to be involved in the activation of MAM, we also measured ADH activities in the cytosols of liver and colon mucosae. Although the livers showed four to five times higher activities of ADH than the colon mucosae, there were no significant differences in the activities of this enzyme between the WT and null groups.

The total cytochrome P-450 contents of liver microsomes from the WT and the null groups were comparable. When CYP2E1 was assayed using either the NDMA demethylase assay or the *p*-NP hydroxylase assay, enzyme activities were significantly less in the liver microsomes from the null mice compared with the WT mice in both males and females. However, it was noted that the microsomes from the null mice showed only 5–9% of the activity of the WT mice in the NDMA demethylase assay, whereas when the *p*-NP hydroxylase assay was used, the apparent CYP2E1 activity in the liver microsomes from the null mice was 30–35% that of microsomes from the WT mice. This indicates that NDMA is a more specific substrate for CYP2E1 than is *p*-NP, and that P-450s other than CYP2E1 participate

in the hydroxylation of *p*-NP in the liver microsomes from the null mice.

With the exception of CYP2B1 and CYP2A5, assays for other P-450 isozymes showed no significant differences between the WT and null groups. In the case of CYP2B1, there was no difference between the null and WT male mice, but the pentoxoresorufin dealkylase activity in liver microsomes from female mice was 2-fold higher in the null group than in the WT group. In the case of CYP2A5, a marked gender difference was observed in the liver microsomal coumarin 7-hydroxylase activity, which was 3-fold higher in female mice than in the male mice. This is similar to the results reported by van Iersel *et al.* (33). Also, this activity was significantly less in the null mice compared with the WT mice, and this difference was especially marked in the female mice. The apparently decreased CYP2A5 activity in liver microsomes from null mice, as measured by coumarin 7-hydroxylase, is no doubt attributable to an overlap of catalytic selectivity between CYP2E1 and CYP2A5, as noted previously by Guengerich and Shimada (36).

**AOM- and MAMAc-induced DNA Guanine Methylation.** The extent of alkylation of DNA guanine to 7-MeG and O<sup>6</sup>-MeG is directly related to the degree of AOM and MAM activation *in vivo* (Fig. 1). Table 2 compares the effects of WT and null status on AOM- and MAMAc-induced DNA guanine alkylation in the liver, colon, kidney, and lung. In all of the WT mouse organs examined, and regardless of gender, the amounts of 7-MeG and O<sup>6</sup>-MeG produced by AOM were similar to those produced by an equimolar dose of MAMAc. This implies that, in the WT mice, almost all of the AOM administered was rapidly metabolized to MAM. In both the WT and null mice, depending on the organ, the level of 7-MeG was 5–7.5 times higher than that of O<sup>6</sup>-MeG, and the differences among various groups were similar whether the statistical comparisons were performed on O<sup>6</sup>-MeG or on 7-MeG levels. Also, in both males and females, the differences between WT and null groups treated with either AOM or MAMAc showed a similar trend, although the levels of O<sup>6</sup>-MeG in the kidneys and lungs showed a significant gender difference. In WT mice, the degree of AOM- and MAMAc-induced DNA alkylation in the lung was 1.6–2.3-fold higher in females than in males, whereas in the kidneys it was 2.4–3-fold higher in the males than in the females. This may be related to the gender-related differences in kidney CYP2E1 levels as reported by others (37–39); the

Table 1 Activities of specific cytochrome P-450 isozymes and ADH in male and female mice with WT or null CYP2E1 status<sup>a</sup>

CYP	WT Male	Null Male	WT Female	Null Female
ADH in colon cytosol (nmol NADH oxidized/min/mg protein)	13.0 ± 4.3	10.7 ± 3.5	6.8 ± 3.4	9.5 ± 3.5
ADH in liver cytosol (nmol NADH oxidized/min/mg protein)	56.7 ± 3.8	48.5 ± 11.7	53.8 ± 11.0	46.9 ± 8.7
Cytochrome P-450 in liver microsomes (nmol P-450/mg protein)	0.66 ± 0.07	0.53 ± 0.09	0.58 ± 0.11	0.44 ± 0.01
NDMA demethylase (nmol HCHO formed/min/mg protein)	2E1 0.62 ± 0.05	0.03 ± 0.03 <sup>b</sup>	0.56 ± 0.07	0.05 ± 0.04 <sup>c</sup>
<i>p</i> -NP hydroxylase (nmol 4-NC formed/min/mg protein)	2E1 0.34 ± 0.04	0.10 ± 0.03 <sup>b</sup>	0.37 ± 0.02	0.13 ± 0.03 <sup>c</sup>
Erythromycin demethylase (nmol HCHO formed/min/mg protein)	3A4 0.69 ± 0.18	0.57 ± 0.11	0.81 ± 0.22	0.55 ± 0.07
Coumarin 7-hydroxylase (nmol 7-OH coumarin formed/min/mg protein)	2A5 0.26 ± 0.06	0.09 ± 0.04	0.82 ± 0.16 <sup>d</sup>	0.37 ± 0.12 <sup>c</sup>
Pentoxoresorufin dealkylase (pmol resorufin formed/min/mg protein)	2B1 17.9 ± 5.5	13.7 ± 4.1	10.5 ± 2.0 <sup>d</sup>	21.1 ± 0.5 <sup>c</sup>
Methoxyresorufin dealkylase (pmol resorufin formed/min/mg protein)	1A2 61.5 ± 7.2	61.0 ± 11.7	54.1 ± 2.2	59.5 ± 1.7
Ethoxyresorufin dealkylase (pmol resorufin formed/min/mg protein)	1A1 34.6 ± 0.8	30.3 ± 3.2	21.7 ± 6.0 <sup>d</sup>	28.4 ± 3.1

<sup>a</sup> Data represent the mean ± SD of three determinations, each derived from a separate mouse.

<sup>b</sup> *P* < 0.05 compared with the corresponding value obtained in the male WT group.

<sup>c</sup> *P* < 0.05 compared with the corresponding value obtained in the female WT group.

<sup>d</sup> *P* < 0.05 compared with the corresponding value obtained in the male WT group.



Table 2 AOM- and MAMAc-induced DNA guanine methylation in various organs of male and female mice with WT or null CYP2E1 status<sup>a</sup>

Group	Liver	Colon	Kidney	Lung
A. 7-MeG levels (pmol 7-MeG/nmol G)				
Male WT AOM	4.895 ± 1.003	0.499 ± 0.243	0.533 ± 0.130	0.048 ± 0.015
Male Null AOM	0.734 ± 0.079 <sup>b</sup>	0.258 ± 0.047 <sup>b</sup>	0.134 ± 0.042 <sup>b</sup>	0.003 ± 0.007 <sup>b</sup>
Male WT MAMAc	5.067 ± 0.505	0.380 ± 0.051	0.558 ± 0.100	0.049 ± 0.008
Male Null MAMAc	3.160 ± 0.290 <sup>c</sup>	1.509 ± 0.384 <sup>c</sup>	0.818 ± 0.100 <sup>c</sup>	0.118 ± 0.019 <sup>c</sup>
Female WT AOM	5.134 ± 0.499	0.598 ± 0.239	0.197 ± 0.036 <sup>d</sup>	0.090 ± 0.027 <sup>d</sup>
Female Null AOM	0.866 ± 0.076 <sup>e</sup>	0.300 ± 0.061 <sup>e</sup>	0.058 ± 0.015 <sup>e</sup>	0.005 ± 0.013 <sup>e</sup>
Female WT MAMAc	6.107 ± 0.650 <sup>f,g</sup>	0.627 ± 0.180	0.187 ± 0.065 <sup>f</sup>	0.105 ± 0.024 <sup>f</sup>
Female Null MAMAc	4.001 ± 0.441 <sup>h,i</sup>	1.563 ± 0.178 <sup>h</sup>	0.422 ± 0.036 <sup>h,i</sup>	0.169 ± 0.020 <sup>h,i</sup>
B. O <sup>6</sup> -MeG levels (pmol O <sup>6</sup> -MeG/nmol G)				
Male WT AOM	0.647 ± 0.142	0.073 ± 0.033	0.074 ± 0.019	0.0094 ± 0.0016
Male Null AOM	0.081 ± 0.014 <sup>b</sup>	0.038 ± 0.004 <sup>b</sup>	0.022 ± 0.003 <sup>b</sup>	0.0054 ± 0.0008 <sup>b</sup>
Male WT MAMAc	0.680 ± 0.067	0.057 ± 0.009	0.082 ± 0.014	0.0098 ± 0.0015
Male Null MAMAc	0.422 ± 0.046 <sup>c</sup>	0.210 ± 0.053 <sup>c</sup>	0.120 ± 0.020 <sup>c</sup>	0.0190 ± 0.0027 <sup>c</sup>
Female WT AOM	0.682 ± 0.073	0.092 ± 0.038	0.031 ± 0.005 <sup>d</sup>	0.0151 ± 0.0031 <sup>d</sup>
Female Null AOM	0.098 ± 0.012 <sup>e</sup>	0.041 ± 0.008 <sup>e</sup>	0.011 ± 0.004 <sup>e</sup>	0.0057 ± 0.0007 <sup>e</sup>
Female WT MAMAc	0.818 ± 0.108 <sup>f,g</sup>	0.092 ± 0.028 <sup>f</sup>	0.032 ± 0.008 <sup>f</sup>	0.0175 ± 0.0029 <sup>f</sup>
Female Null MAMAc	0.529 ± 0.059 <sup>h,i</sup>	0.216 ± 0.028 <sup>h</sup>	0.060 ± 0.005 <sup>h,i</sup>	0.0254 ± 0.0023 <sup>h,i</sup>

<sup>a</sup> Mice were treated s.c. with either AOM (14 mg/kg) or MAMAc (25 mg/kg) and sacrificed 6 h later. The DNA isolated from liver, colon mucosa, kidney, or lung was analyzed for 7-MeG and O<sup>6</sup>-MeG. Data represent mean ± SD of six determinations, each derived from a separate mouse. Group means were compared among the groups using ANOVA followed by Fisher's protected *t* test.

<sup>b</sup> *P* < 0.05 compared with the corresponding value obtained in the male-WT-AOM group.

<sup>c</sup> *P* < 0.05 compared with the corresponding value obtained in the male-WT-MAMAc group.

<sup>d</sup> *P* < 0.05 compared with the corresponding value obtained in the male-WT-AOM group.

<sup>e</sup> *P* < 0.05 compared with the corresponding value obtained in the female-WT-AOM group.

<sup>f</sup> *P* < 0.05 compared with the corresponding value obtained in the male-WT-MAMAc group.

<sup>g</sup> *P* < 0.05 compared with the corresponding value obtained in the female-WT-AOM group.

<sup>h</sup> *P* < 0.05 compared with the corresponding value obtained in the female-WT-MAMAc group.

<sup>i</sup> *P* < 0.05 compared with the corresponding value obtained in the male-Null-MAMAc group.

expression of CYP2E1 in mouse kidney is male specific and is regulated by testosterone.

In male WT mice, DNA guanine alkylation was highest in the liver and lowest in the lung. The levels of O<sup>6</sup>-MeG in liver DNA were 8–10 times higher than in the colon mucosa or kidney and ~70 times higher than in the lung. In mice treated with AOM, as well as in those treated with MAMAc, the O<sup>6</sup>-MeG levels in liver DNA were lower in the null group than in the WT group. However, in the case of the animals treated with AOM, the alkylation of liver DNA guanine to O<sup>6</sup>-MeG was 87% lower in the null mice than in the WT mice, whereas in the case of MAMAc treatment, it was only 38% lower in the null mice, indicating that the absence of CYP2E1 influenced the activation of AOM to a much greater degree than the activation of MAM. That liver DNA guanine alkylation occurred at all in the AOM-treated null mice indicates the participation of enzymes other than CYP2E1 in the activation of AOM, although the extent of such participation may be minor. The significantly lesser effect of null CYP2E1 status on liver DNA alkylation in mice treated with MAMAc suggests that there is a major involvement of enzymes other than (or in addition to) CYP2E1, perhaps ADH, in the metabolism of MAM in mouse liver.

As in the liver, DNA guanine alkylation of the colon, kidney, and lung was always lower in the null mice compared with the WT mice when AOM was used as the carcinogen (Table 2). The greatest difference was observed in the livers, where 7–8 times less alkylation occurred in the null *versus* the WT mice and the least in the colons, in which 1.9–2.2 times less alkylation occurred. When MAMAc was used as the carcinogen, however, increases in DNA guanine alkylation were noted in the colons, kidneys, and lungs of the male and female null mice compared with the WT mice. These increases were 3.7-, 1.5-, and 1.9-fold, respectively, in the colons, kidneys, and lungs of male mice. Similar increases in DNA guanine alkylation in these organs were observed in female null mice.

**CYP2E1 Status and Colonic ACF Formation Induced by AOM and MAMAc.** As described above, decreased alkylation of DNA guanine to O<sup>6</sup>-MeG was observed in the colons of the null mice

compared with the WT mice when AOM was used as the carcinogen, but increased guanine alkylation was observed in the colonic DNA of the null mice when MAMAc was used as the carcinogen. This suggested that although the carcinogenicity of AOM for the colon would be decreased by the null CYP2E1 status with respect to the WT status, the colon carcinogenicity of MAMAc might be enhanced. As an initial test of this prediction, we compared the ability of AOM and MAMAc to induce colonic ACF in WT and null mice. ACF represent precancerous lesions in animal models and in humans (5–7).

The study on ACF formation consisted of two consecutive parts. In the first part, 10 each male and female WT and null mice were treated s.c. with 10 mg/kg AOM or 0.9% solution of NaCl (vehicle controls). Although the intent in this part of the study was to repeat the AOM dosing and kill the mice 3 weeks after the last dose, 10 mg/kg (135 μmol/kg) proved to be lethal to 8 and 4 mice in the male and female WT groups, respectively, in the first week after dosing. No further deaths were observed in any of the groups in the next 2 weeks. In the second part of the study, the original intent was to treat the mice (distributed as in the first part of the study) with an amount of MAMAc equimolar to the AOM; however, because of the toxicity observed with AOM, the dose of MAMAc was lowered to 9 mg/kg (68 μmol/kg). As in the first part, the mice were killed 3 weeks after dosing, and colonic ACF was determined. No toxicity or evidence of ACF was found in any of the vehicle controls.

As shown in Table 3, in the AOM-treated WT group, colonic ACF were present in all mice that survived until sacrifice (2 of 10 males and 6 of 10 females). In the AOM-treated null group, all of the males and all of the females survived the duration of the study, but colonic ACF were detectable in only 30% of the male mice and in none of the female mice. These results indicate that the CYP2E1-null status confers protection against the toxicity of AOM and its colon tumorigenicity in mice and are consistent with the results of the assays of AOM-induced colon DNA guanine alkylation.

As also shown in Table 3, 3 weeks after a single dose of MAMAc, which was one-half the molar amount of the AOM dose, survival was

Table 3 AOM- and MAMAc-induced colonic ACF formation in male and female mice with WT or null CYP2E1 status<sup>a</sup>

Carcinogen	Group	Survival	ACF incidence <sup>b</sup>	No. of ACF/colon <sup>c</sup>
AOM	WT-male	2/10	2/2	3.0 ± 1.0
AOM	Null-male	10/10	3/10	0.5 ± 0.3 <sup>d</sup>
AOM	WT-female	6/10	6/6	3.0 ± 0.4
AOM	Null-female	10/10	0/10	0 <sup>e</sup>
MAMAc	WT-male	10/10	3/10	0.4 ± 0.2
MAMAc	Null-male	10/10	8/10	1.8 ± 0.5 <sup>d</sup>
MAMAc	WT-female	10/10	2/10	0.3 ± 0.2
MAMAc	Null-female	10/10	6/10	1.8 ± 0.6 <sup>e</sup>

<sup>a</sup> Mice were treated with a single s.c. injection of either AOM (10 mg/kg) or MAMAc (9 mg/kg) and sacrificed 3 weeks later for colonic ACF assessment.

<sup>b</sup> Number of colons with ACF over total number of colons scored.

<sup>c</sup> Number of ACF in the distal 5 cm of the colon. All values shown are the mean ± SE.

<sup>d</sup>  $P < 0.05$  compared with the WT-male group treated with the same carcinogen.

<sup>e</sup>  $P < 0.05$  compared with the WT-female group treated with the same carcinogen.

100% in all groups. However, compared with the WT mice, null mice showed very significant increases in the incidence of colonic ACF (from 3 of 10 to 8 of 10 in males and from 2 of 10 to 6 of 10 in females) as well as in the number of ACF per colon (from 0.4 to 1.8 in males and from 0.3 to 1.8 in females). These results indicate that CYP2E1-null status would increase the carcinogenicity of MAMAc and are again consistent with the results from the assay of MAMAc-induced colon DNA guanine alkylation.

## DISCUSSION

Bioengineered animal models that lack the ability to express specific enzymes have proved extremely valuable in the evaluation of metabolic pathways responsible for the activation or detoxication of toxic agents and carcinogens (40–43). The CYP2E1-null mouse is an excellent model with which to investigate the role of metabolism in chemical carcinogenesis because it has no phenotypic or pathological abnormalities. The CYP1A, CYP2A, CYP2B, CYP2C, and CYP3A subfamilies are expressed at normal levels in the livers of null mice, whereas CYP2E1 protein expression is completely absent (40). The use of CYP-null mice to determine metabolic pathways is preferable to the use of metabolic inhibitors *in vivo*, because it is extremely difficult to prove that inhibitors are affecting only one enzyme. Indeed, CYP2E1-null mice have become a powerful tool for directly and accurately defining the role of the enzyme in the metabolism and toxicity of several important CYP substrates, including benzene, acetaminophen, and carbon tetrachloride (40–43).

In the present work, we have examined the effects of CYP2E1 deficiency on AOM- and MAMAc-induced DNA guanine methylation in the liver, colon, kidney, and lung and on the colonic formation of ACF, which represent early preneoplastic lesions (5). The results showed that in null mice treated with AOM,  $O^6$ -MeG levels in all organs were reduced compared with AOM-treated WT mice. However, in null mice treated with MAMAc, the degree of DNA alkylation was reduced in the liver but increased in the colon and other extrahepatic organs compared with WT mice. These results indicate that although the carcinogenicity of AOM for the colon may be reduced in null mice compared with AOM-treated WT mice, the colon carcinogenicity of MAMAc might be increased. The results of the ACF studies supported this expectation; the incidence and multiplicity of colonic ACF were decreased in the AOM-treated null mice compared with the WT, whereas an increase occurred in the MAMAc-treated null mice relative to the WT mice.

The lower amount of DNA guanine methylation in all organs examined of AOM-treated null mice compared with WT mice (Table 2) treated in the same way indicates a major involvement of CYP2E1 in the hydroxylation of AOM to MAM (Fig. 1). Because the level of guanine methylation in the AOM-treated CYP2E1-null mice is not zero but a

finite amount (Table 2), and because AOM is not a direct-acting methylating agent, the participation of enzymes other than CYP2E1 is also indicated in this metabolic reaction. As in the case of NDMA (44), CYP2A5 seems a good candidate for this role. In this respect, it is informative to note the similarities between the metabolic activation of AOM and of NDMA (9, 45). Both AOM and NDMA are metabolically hydroxylated on a methyl group in a reaction catalyzed by CYP2E1; the former to MAM (Fig. 1), the latter to *N*-methyl(hydroxymethyl)nitrosamine. However, although the highly reactive  $\alpha$ -hydroxy metabolite derived from NDMA has a half-life of only a few seconds (reviewed in Ref. 46), MAM is a relatively long-lived species, with a half-life of ~12 h (11, 12). Thus, although NDMA is primarily a liver carcinogen because its highly reactive  $\alpha$ -hydroxy metabolite reacts with macromolecules mostly at the organ site of its formation, the much longer half-life of MAM allows it to be distributed to other organs, such as the colon and kidneys, to be further activated at these sites.

In MAMAc-treated CYP2E1-null mice, the degree of DNA guanine methylation in the colon and kidneys is increased compared with MAMAc-treated WT mice; at the same time, DNA guanine methylation in the liver of the null mice is decreased by ~38% (Table 2). This suggests that although CYP2E1 is involved in the liver activation of MAM, the involvement is minor; the majority of the activation is carried out by enzymes other than CYP2E1. Enzymes other than CYP2E1 are also responsible for MAM activation in extrahepatic organs such as the colon, kidney, and lung. The identity of these enzymes is unknown, although evidence for the involvement of ADH (12), choline dehydrogenase (47), and prostaglandin synthase (48) has been reported. Because less MAM is metabolized in the liver of the CYP2E1-null mice, more of the carcinogen becomes available for activation, by other enzymes, at extrahepatic sites. This results in increased DNA guanine methylation and increased ACF formation, as observed. In the case of AOM, liver CYP2E1 appears to be the main (though not the only) enzyme involved in its activation to MAM. Thus, with respect to the AOM-treated WT mice, the AOM-treated CYP2E1-null mice showed significant decreases in DNA guanine methylation in the liver and all other organs examined, as well as decreases in ACF formation.

AOM and MAM are not generally regarded as human carcinogens. However, MAM occurs naturally as the aglycone of cycasin, the product of the palm *Cycas circinalis* (49), and the facile formation of AOM during the oxidation of methylamine has been described (50). Because the presence of these or chemically similar compounds in the environment cannot be ruled out, there is a need for more thorough knowledge of the enzymes involved in their activation and detoxication.

Hepatic CYP2E1 activity is known to increase or decrease in response to numerous xenobiotics and physiological conditions (51–53). It can be expected, therefore, that increases or decreases in CYP2E1 activity levels will also affect the metabolism of AOM and MAM, which in turn will influence tumorigenesis induced by these carcinogens. In the present work, we show that the metabolic activation of these chemically and metabolically related colon carcinogens is affected quite differently in CYP2E1-WT and CYP2E1-null mice. In the case of AOM, null CYP2E1 status is manifested by decreased DNA guanine alkylation in colon DNA and decreased ACF formation with respect to the WT. In the case of MAMAc, null status is characterized by increased DNA guanine alkylation in colon DNA and increased ACF formation. On the basis of these results, we suggest that caution be used in the interpretation of studies of chemopreventive agents using the AOM or MAMAc colon cancer model at the tumor initiation stage. If the agents modify the activity of CYP2E1 to a sufficient degree, diametrically opposite results might be obtained, depending on which of the two chemically and metabolically related colon carcinogens is administered.

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