Metabolism of $N$-Nitrosomethyl-$n$-amylamine by Microsomes from Human and Rat Esophagus

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ABSTRACT

Asymmetric dialkylnitrosamines induce esophageal cancer in rats and hence might be involved in the etiology of this cancer in humans. As a test of this hypothesis, we examined whether nitrosamines can be activated by segments of human esophagus and by microsomes of human and rat esophagus and liver. Specimens of 8 human esophagi were test of this hypothesis, we examined whether nitrosamines can be activated by segments of human esophagus and by microsomes of human and rat esophagus and liver. Specimens of 8 human esophagi were tested for the metabolism of $N$-nitrosomethyl-$n$-amylamine (NMAA). Hydroxyl-NMAA yields were determined by gas chromatography-thermal energy analysis and were insignificant except for those of 5-hydroxy-NMAA, which were low. Microsomes were prepared from 4 batches of human esophagi and samples with 0.6 mg protein were incubated for 20 min with NMAA and cytochrome P-450 cofactors. We determined hydroxy-NMAA as before and aldehydes by high-performance liquid chromatography of their 2,4-dinitrophenylhydrazones. Incubation of these microsomes with 12 mM NMAA yielded mean values of 0.64 nmol formaldehyde ("demethylation"). 0.21 nmol pentaldehyde ("depropylation") and 0.56 nmol total hydroxy-NMAAs/min/mg protein. Metabolite yields under various conditions were determined, including a demonstration that carbon monoxide inhibited 81% of NMAA demethylation, indicating that cytochrome P-450 enzymes were involved. We also examined $N$-nitrosodimethylamine (NDMA) demethylation by the same microsomes. Rat esophageal microsomes dealkylated NMAA and NDMA similarly to human esophageal microsomes, but with 2-6 times and twice the activity, respectively. Human and rat esophageal microsomes demethylated 6 mM NMAA 18-20 times as rapidly as they demethylated 5 mM NDMA, in contrast to liver microsomes of these species, which demethylated 6 mM NMAA only 0.9-1.4 times as rapidly as they demethylated 5 mM NDMA. However, liver microsomes of both species were more active than esophageal microsomes for NDMA demethylation. The occurrence of NMAA demethylation and (to a lesser extent) depropylation with both human and rat esophageal microsomes is important because these are the activating reactions, and suggests that both human and rat esophagus contain P-450 enzymes that specifically dealkylate asymmetric dialkylnitrosamines.

INTRODUCTION

In Western countries, esophageal cancer is associated with tobacco and alcohol use (3), and the most likely initiating agents for esophageal cancer are nicotine-related nitrosamines in tobacco smoke (3). Esophageal cancer is the seventh commonest cancer worldwide (4) and shows very high rates among blacks in Transkei, South Africa, Turkomani in Iran, and Chinese in Linxian county, China (5). In these areas, environmental and nutritional factors are important in the etiology (6), and esophageal cancer could be initiated by nitrosamines formed by gastric nitrosation of food-derived amines (7).

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3 The abbreviations used are: NDMA, $N$-nitrosodimethylamine; GC-TEA, gas chromatography with detection by thermal energy analysis; HO, hydroxy; NMAA, $N$-nitrosomethylamylamine.

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lism can reflect that by the whole tissue. These microsomes also catalyzed formaldehyde (HCHO) and pentaldehyde formation from NMAA (demethylation and depentylation, respectively). These reactions should yield, respectively, a pentylating and a methylyating agent, which might alkylate DNA and initiating cancer (Fig. 1). Using inhibitory monoclonal antibodies, we identified the P-450 isozymes in rat liver microsomes that are responsible for at least part of NMAA metabolism at each of 6 positions (22). Because nitrosamines are potent carcinogens and humans are exposed to them (23), we studied NMAA metabolism by human esophageal segments and by human and rat esophageal microsomes.

MATERIALS AND METHODS

Chemicals. NMAA was synthesized with >99% purity as determined by GC-TEA (19). NDMA was prepared similarly. The 2- to 5-HO-NMAAs were synthesized as before (19–21). Experiments with nitrosamines, which are volatile carcinogens, were conducted with due precautions.

Studies with Human Esophageal Segments. Esophagei were obtained at the University of Maryland from autopsies of apparently healthy individuals of various ages and of both sexes. Within 6 h of death, the tissues were transported on ice over <1 h to a tissue culture laboratory. From each esophagus, 2 g of 2–3-mm segments (including the mucosa) were incubated with shaking in 10 ml CMRL-1066 culture medium with 23 and 300 μM NMAA at 37°C in closed 50-ml tubes. The medium was extracted with dichloromethane and analyzed for HO-NMAAs by GC-TEA (19, 20).

Isolation of Human Esophageal and Liver Microsomes. Samples 1–3 of esophagus were batches of 10–20-g segments, each combined from several adults and obtained at the University of Maryland or elsewhere (see "Acknowledgments"). Sample 4 of esophagus comprised 5 g of segments from a black woman in her 70s. Sample 1 of human liver was from an adult black woman. These samples were stored at −80°C and mailed to Omaha in dry ice. Liver samples 2 (from an adult man) and 3 (from a young girl) were obtained from the University of Nebraska Medical Center liver transplant program.

To isolate the microsomes, each batch of esophageal segments was suspended in 40–80 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 15.4 mg diithiothreitol/liter ("buffer A") and homogenized in a Virtis homogenizer (Fisher Scientific Co., Gardena, CA) with new blades, which was run 3 times for 45 s at top speed, with intermediate cooling in ice for 2 min. The homogenate was filtered through 1 layer of gauze and rehomogenized in a 60-ml Potter-Elvehjem homogenizer with a Teflon piston (driven by a Con-Torque power unit [Eberbach, Ann Arbor, MI]), which was passed twice down and up with intermediate cooling in ice. Each liver sample (20 g) was homogenized in buffer A with the Potter-Elvehjem homogenizer. All homogenates were centrifuged at 9,000 × g for 30 min and then at 105,000 × g for 60 min. The microsomal fractions were resuspended in 5 ml buffer A, subdivided into several fractions, and stored at −80°C. The esophageal samples contained about 10 mg protein/5 g tissue as determined by the Lowry (24) method with bovine serum albumin as the standard.

Isolation of Rat Esophageal and Liver Microsomes. The tissues were taken from adult male Sprague-Dawley rats. Of the esophageal microsomes, batch 1 was prepared from 50 rat esophagi that had been stored for 1–2 years at −80°C, and batches 2 and 3 were each prepared from 50 esophagi frozen on dry ice when dissected and mailed in dry ice by Bioproducts for Science (Indianapolis, IN). The esophagi were thawed, stripped of outer connective tissue with a forceps, and homogenized in buffer A with the Potter-Elvehjem homogenizer, with 6 passes down and up of the piston. Esophageal microsomes were isolated and stored at −80°C as for the human microsomes. Each batch contained 17–25 mg protein. Liver microsomes were prepared from untreated rats as before (22).

Microsomal Incubations with Nitrosamines. Under the standard conditions, microsomes containing 0.6 mg protein were incubated for 20 min at 37°C with NMAA or NDMA in 0.5 ml of a medium containing 100 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl2, 2 mM NADP, 10 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and (when aldehydes were determined) 5 mM semicarbazide-HCl (22). Final pH was 7.4 (HO-NMAA assays) or 7.0 (aldehyde assays). To determine HO-NMAAs, reactions were stopped by adding 0.2 ml 10% HClO4, the HO-NMAAs were extracted with CH2Cl2, and the extracts were concentrated and analyzed by GC-TEA (22). All HO-NMAA results were corrected for losses during extraction and gas chromatography as before (19–22).

Aldehydes were determined by a modification of Farrelly's (25) method as before (22). In brief, reactions were stopped by adding Ba(OH)2 and ZnSO4. After centrifugation, each supernatant was reacted with 2,4-dinitrophenylhydrazine-HCl. The hydrazones were extracted into iso-octane. The iso-octane phase was back-extracted with acetonitrile. This extract was concentrated to 0.2 ml, and 0.1 ml was subjected to high-performance liquid chromatography on a C-18 column developed with acetonitrile-water, 3:1. Retention times were 3.1 min for the HCHO and 7.2 min for the pentaldehyde hydrazone. This method produces quantitative recoveries of HCHO and 74% recoveries of pentaldehyde (22), the results of which were corrected for this loss. A ± B always signifies mean ± SE. Student's t test was used in all tests of significance.

RESULTS

Segments of 8 human esophagi were incubated for 6 h with 23 and 300 μM NMAA. As in our previous studies on whole rat esophagi (20), the stable HO-NMAAs were determined but not the aldehydes. With 23 μM NMAA, the 5-HO-NMAA yield was 0.17 ± 0.04% of the NMAA (mean ± SE), significantly (P < 0.01) different from zero, and the yields of 2-, 3-, and 4-HO-NMAAs were <0.04% and not significant. When 300 μM NMAA was used, the yields of 2-, 3-, and 4-HO-NMAA were not significantly raised above the low but detectable levels of these nitrosamines (0.002–0.005% of the NMAA) observed in blanks with NMAA but without tissue, and the yield of 5-HO-NMAA was 0.020 ± 0.005% of the NMAA, significantly (P < 0.01) exceeding the blank value of 0.001%. (These blank values would be undetectable with 23 μM NMAA.) We did not check whether incubation of human esophageal segments without NMAA produced a GC-TEA peak at the position where 5-HO-NMAA is eluted, but no such peak was detected in similar experiments with rat esophagus or liver slices incubated for 2 h without NMAA. We conclude that segments of human esophagus did not produce significant amounts of the HO-NMAAs from NMAA, except for 5-HO-NMAA.

The human esophagi were removed up to 6 h after death, usually while the bodies were refrigerated, and another 1–2 h elapsed before incubation with NMAA was begun. The low HO-NMAA yields with the esophageal segments might have been due to this delay, which could have killed the metabolizing cells. As a model for this situation, HO-NMAA production was compared in rat esophagi that were incubated for 3 h with 300
μM NMAA (a) immediately after the rats were sacrificed; or (b) after storage for 18 h at 6°C as dissected esophagi or in the carcases. Total HO-NMAA yield/3 h/100 mg tissue was 1.1–1.5% for the freshly incubated tissue and 0.7–0.8% for the stored preparations. These results suggest that the human esophagi would have lost some but not all of their activity before the incubation with NMAA.

In the experiments with microsomes, both HO-NMAA and aldehyde metabolites were measured (21, 22). Table 1 shows the metabolite yields when human esophageal microsomes were incubated with 6 or 12 mM NMAA or with 5 or 10 mM NDMA. A 20-min incubation was used because NMAA metabolism by rat liver microsomes is linear for this time (21, 22). NMAA produced 2- to 5-HO-NMAA, with a predominance of 2-HO-NMAA and a mean total HO-NMAA yield of 0.56 nmol/min/mg protein when 12 mM NMAA was used. For each of the tests in Table 1, mean total HCHO yield was significantly (P < 0.01) greater than the corresponding mean blank value with microsomes but without NMAA. Mean total HCHO yield from 6 mM NMAA was 1.7 and 2.5 times the mean blank for microsomes with 0.6 and 1.2 mg protein, respectively. Mean net HCHO formation was 0.40 (from 6 mM NMAA) and 0.64 (from 12 mM NMAA) nmol/min/mg under the standard conditions (Table 1, Tests 1–7), and was 0.56 nmol/min/mg for 6 mM NMAA and microsomes with 1.2 mg protein (Table 1, Test 8). Pentaldehyde yields were 0.04 (from 6 mM NMAA) and 0.21 (from 12 mM NMAA) nmol/min/mg protein under the standard conditions and were significantly different from zero (P < 0.01) for the tests with 12 mM NMAA. Carbon monoxide did not affect HCHO yield in the blank but inhibited net HCHO formation from 6 mM NMAA by 81% (Table 1, Test 9).

The human esophageal microsomes showed little metabolism of NDMA, with mean net yields of only 0.02 and 0.11 nmol HCHO/min/mg protein from 5 and 10 mM NDMA, respectively (Table 1, Tests 11–16). These results were significantly (P < 0.01) different from the blanks only in the tests with 10 mM NDMA.

Rat esophageal microsomes metabolized 6 mM NMAA to give mean yields of 0.73 nmol HCHO, 0.42 nmol pentaldehyde, and only 0.06 nmol of total HO-NMAAs/min/mg (Table 2). The ratio of the mean activity of these microsomes (Table 2) to that of human esophageal microsomes (Table 1) was about 2 for the demethylation and 6 for the depentylation of 6 mM NMAA, and was 2 for the demethylation of 5 and 10 mM NMAA. Batch 1 of the rat esophageal microsomes, in which the esophagi were stored for 1–2 years before the microsomes were isolated, gave results similar to those for batches 2 and 3, for which the esophagi were worked up upon receipt, indicating that intact tissue can be stored without loss of microsomal activity. In studies on human liver microsomes (Table 3), sample 1 showed considerable demethylation and depentylation but little 2- to 5-hydroxylation of NMAA. Samples 2 and 3 showed considerable demethylation, depentylation, and 4-hydroxylation of NMAA. The human liver microsomes were 15–60% as active as rat liver microsomes (Table 2) for the demethylation of 5 mM NDMA.

DISCUSSION

Human and rat esophageal microsomes behaved similarly in that they showed ratios of 20 and 18, respectively, for mean demethylation of 6 mM NMAA/mean demethylation of 5 mM NDMA (Tables 1 and 2), compared to ratios of only 1.4 and 0.9 for human and rat liver microsomes, respectively (Tables 2 and 3). Hence, for both species, esophageal microsomes showed a preferential demethylation of NMAA, whereas liver microsomes showed a preferential demethylation of NDMA. In con-
N-NITROSOMETHYLAMYLAMINE METABOLISM IN ESOPHAGUS

Table 2 Metabolism of 6 mM NMAA and 10 mM NDMA by esophageal and liver microsomes from uninduced adult male Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Test no.</th>
<th>Name</th>
<th>Batch</th>
<th>Conc. (mm)</th>
<th>Total HO-NMAAs</th>
<th>HCHO*</th>
<th>Pentaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Esophagus 1</td>
<td>NMAA</td>
<td>6</td>
<td>0.05 ± 0.01</td>
<td>0.76 ± 0.13</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>Esophagus 2</td>
<td>NMAA</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>3</td>
<td>Esophagus 3</td>
<td>NMAA</td>
<td>6</td>
<td>0.10 ± 0.00</td>
<td>0.42 ± 0.07</td>
<td>0.68 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>Liver</td>
<td>1</td>
<td>6</td>
<td>0.05 ± 0.01</td>
<td>1.01 ± 0.01</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>Esophagus 2</td>
<td>NDMA</td>
<td>5</td>
<td>NR</td>
<td>1.71 ± 0.01</td>
<td>1.18 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>Esophagus 2</td>
<td>NDMA</td>
<td>10</td>
<td>NR</td>
<td>0.04 ± 0.03</td>
<td>NR</td>
</tr>
<tr>
<td>7</td>
<td>Liver</td>
<td>1</td>
<td>NDMA 5</td>
<td>NR</td>
<td>0.20 ± 0.05</td>
<td>NR</td>
</tr>
<tr>
<td>8</td>
<td>Liver</td>
<td>1</td>
<td>NDMA 10</td>
<td>NR</td>
<td>1.25 ± 0.20</td>
<td>NR</td>
</tr>
</tbody>
</table>

* Mean blank in absence of nitrosamine was 0.58 (for esophagus) and 0.35 (for liver) nmol/min/mg protein.

Table 3 Metabolism of NMAA and NDMA by human liver microsomes

<table>
<thead>
<tr>
<th>Test no.</th>
<th>Name</th>
<th>Conc. (mm)</th>
<th>Donor no.</th>
<th>2-HO-NMAA</th>
<th>3-HO-NMAA</th>
<th>4-HO-NMAA</th>
<th>5-HO-NMAA</th>
<th>Net HCHO*</th>
<th>Pentaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NMAA</td>
<td>6</td>
<td>1</td>
<td>0.09 ± 0.01</td>
<td>0.06 ± 0</td>
<td>0.05 ± 0</td>
<td>0.03 ± 0</td>
<td>0.65 ± 0.1</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>NMAA</td>
<td>6</td>
<td>2</td>
<td>0.13 ± 0.01</td>
<td>0.40 ± 0.02</td>
<td>1.13 ± 0.02</td>
<td>0.02 ± 0</td>
<td>0.68 ± 0.23</td>
<td>1.19 ± 0.20</td>
</tr>
<tr>
<td>3</td>
<td>NMAA</td>
<td>6</td>
<td>3</td>
<td>0.09 ± 0.01</td>
<td>0.32 ± 0.04</td>
<td>1.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.52 ± 0.01</td>
<td>1.17 ± 0.08</td>
</tr>
<tr>
<td>4</td>
<td>NMAA</td>
<td>12</td>
<td>1</td>
<td>0.26 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>1.37 ± 0.17</td>
<td>0.85 ± 0.27</td>
</tr>
<tr>
<td>5</td>
<td>NMAA</td>
<td>12</td>
<td>2</td>
<td>0.30 ± 0.02</td>
<td>0.94 ± 0.07</td>
<td>2.09 ± 0.24</td>
<td>0.09 ± 0.01</td>
<td>1.17 ± 0.46</td>
<td>2.31 ± 0.34</td>
</tr>
<tr>
<td>6</td>
<td>NMAA</td>
<td>12</td>
<td>3</td>
<td>0.15 ± 0.02</td>
<td>0.45 ± 0.01</td>
<td>1.02 ± 0.04</td>
<td>1.06 ± 0.01</td>
<td>0.59 ± 0.02</td>
<td>2.08 ± 0.30</td>
</tr>
<tr>
<td>7</td>
<td>NDMA</td>
<td>5</td>
<td>1</td>
<td>0.19 ± 0.01</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>8</td>
<td>NDMA</td>
<td>5</td>
<td>2</td>
<td>0.77 ± 0.07</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>9</td>
<td>NDMA</td>
<td>5</td>
<td>3</td>
<td>0.38 ± 0.18</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>10</td>
<td>NDMA</td>
<td>10</td>
<td>1</td>
<td>0.75 ± 0.09</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>11</td>
<td>NDMA</td>
<td>10</td>
<td>2</td>
<td>1.12 ± 0.03</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>12</td>
<td>NDMA</td>
<td>10</td>
<td>3</td>
<td>0.83 ± 0.09</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

* Mean blank in absence of nitrosamines was 0.42 nmol/min/mg protein.

Rat esophageal microsomes produced little of the HO-NMAAs (Table 2), whereas the whole rat esophagus produced large amounts of 2-, 3-, and 4-HO-NMAA from NMAA. Therefore, the microsome preparation did not account for the entire activity of rat and perhaps also human esophagus.

We might have underestimated the contribution of P-450 2E1 to NMAA and NDMA metabolism because we used glycerol in the medium and semicarbazide in the microsomal incubations, both of which competitively inhibit nitrosamine metabolism by 2E1 (29), though omission of glycerol had no effect in 1 test (Table 3, Footnote c). Only a trace of P-450 2E1 was observed in a preliminary study of immunoblots of solubilized rat esophageal microsomes that were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with a monoclonal antibody (1-98-1) to 2E1.5 Accordingly, it is important to identify the esophageal-specific P-450 in rat esophagus and determine whether a homologous P-450 occurs in human esophagus.

The activity of human esophageal microsomes was one-half (for NMAA demethylation) and one-sixth (for NMAA depentylation) of that for rat esophageal microsomes (Tables 1 and 2). Nevertheless, the similar pattern of metabolism by these 2 types of microsomes suggests that they contain homologous esophageal-specific P-450 isozymes that can activate NMAA and, perhaps, other asymmetric dialkyl and cyclic nitrosamines that induce esophageal cancer in rats (3). However, we do not yet know whether human tissues other than esophagus and liver can dealkylate NMAA. Also, we cannot assume that nitrosamine activation by the endoplasmic reticulum leads to DNA...
alkylation in the nucleus because (a) active intermediates might decompose before they reach the nucleus; and (b) aldehydes are produced from nitrosamines not only by \( \alpha \)-hydroxylation (Fig. 1) but also by denitrosation, which is 30% of total DNA metabolism in rat liver (30).

Demethylation exceeded depentylation of NMAA for both human and rat esophageal microsomes (Tables 1 and 2). However, demethylation of NMAA should lead to DNA pentenylation, which has been searched for but not detected in the esophagus of NMAA-treated rats. Depentylation should lead to DNA methylation, and the depentylation of NMAA by rat esophageal microsomes (Table 2) is consistent with the observed methylation of esophageal DNA bases by NMAA (31). Therefore, only NMAA depentylation may present a carcinogenic hazard. Depentylation by human esophageal microsomes was significant for 12 mM NMAA, suggesting that DNA methylation would occur if there was exposure to NMAA. Accordingly, our results support the view that NMAA or other methyalkynitrosamines contribute to the etiology of human esophageal cancer.

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