Metabolism of N-Nitrosamines by Cultured Human and Rat Esophagus

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ABSTRACT

The metabolism of several N-nitrosamines (N-nitrosodimethylamine, N-nitrosoethylmethyamine, N-nitrosodimethylamine, N-nitrosobenzylmethylamine, and N-nitrosopyrrolidine) in cultured human and rat esophagus has been investigated by measuring (a) CO2, (b) metabolites with an oxo group, and (c) metabolites bound to DNA. Both acyclic and cyclic N-nitrosamines were metabolized by rat esophagus. The highest level of metabolite binding was seen with N-nitrosobenzylmethylamine, an organotrophic carcinogen for the rat esophagus. The binding level was about 100-fold higher than in human esophagus. This compound methylated rat esophageal DNA at positions 7 and O6 of guanine. The level of benzylation in rat was one-tenth the level of methylation. Formation of benzaldehyde exceeded that of formaldehyde plus CO2 by a factor of six, indicating that the methylene group was preferentially oxidized. N-Nitrosoethylmethyamine, another unsymmetrical N-nitrosamine, was preferentially oxidized by rat esophagus in the ethyl group, as shown by higher formation of CO2 and acetaldehyde from the compound labeled in the ethyl group. The highest binding level to DNA from this compound was observed with the methyl group. No binding was detected to human esophagus. N-Nitrosopyrrolidine was oxidized by both rat and human esophagus in the a position, as measured by the formation of 2,4-dinitrophenylhydrazine derivative of 4-hydroxybutanalan. Binding of metabolites of N-nitrosopyrrolidine to DNA was detected only in rat esophagus. As measured by the formation of both CO2 and formaldehyde, N-nitrosodimethylamines was metabolized by both human and rat esophagus. While most of the radioactivity associated with DNA was found to be incorporated into guanine and adenine, methylation of the guanine positions 7 and O6 was detected by chromatography of the hydrolyzed rat DNA. The results indicate significant quantitative and perhaps qualitative differences between cultured rat and human esophagus in their ability to activate N-nitrosamines, although unknown physiological differences after culture may contribute to this difference.

INTRODUCTION

Epidemiological studies of esophageal cancer in humans indicate that nutritional and environmental factors are of importance (13, 22, 43, 44). An increased risk for developing esophageal cancer has been correlated with tobacco smoking (43), consumption of alcoholic beverages (41, 42), and certain plant products (44), products which are frequently contaminated with N-nitroso compounds (30). Carcinoma of the esophagus has been induced in experimental animals by several N-nitrosamines (10). An animal model in rats using BMNA, the most potent known inducer of esophageal cancer, has been developed (12, 37). Since environmental N-nitrosamines require metabolic activation to react with cellular macromolecules and their organotropism is generally unrelated to their route of administration, the study of their pharmacokinetics and metabolic activation in potential target tissues is important (23, 24). For example, tissue slices of the mucosal lining of rat esophagus have been shown to activate both DMNA and BMNA into metabolites which alkylate cellular DNA, the major alklylation product for both N-nitrosamines being 7-MeGua (11).

We have reported previously that cultured human esophagi activate DMNA but not NPY into DNA-binding metabolites (16). With the use of an explant culture system developed for rat and human esophagus (16, 38), we have expanded our previous study to a series of N-nitrosamines, DMNA, EMNA, DENA, NPY, and BMNA. Of these compounds, however, only DENA and BMNA induced esophageal cancer in experimental animals, while EMNA and NPY were used in this study to determine the relative importance of the 2 different oxidative pathways in esophageal tissues. The metabolism was measured by determination of the amount of metabolites bound to DNA, alkylation pattern of DNA, and quantitation of metabolites released into the culture media.

MATERIALS AND METHODS

Male CD rats (5 to 6 weeks old; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were killed by asphyxiation, and the esophagi were removed. Explants (3 sq mm) were prepared and cultured without serum as described previously (38). Nontumorous human esophageal tissues were obtained at the time of surgery (HE230, 56-year-old male) and immediate autopsy (HE223, 20-year-old male; HE226, 16-year-old female; HE227, 65-year-old female; HE228, 47-year-old male; HE235, 20-year-old male; HE246, 29-year-old male; HE247, 33-year-old male; and HE252, 22-year-old female), immersed in L-15 tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.), and kept at 4°C for 3 to 8 hr until cultured as described previously (16, 40). The rat esophagus was cultured for either 1 or 7 days, and the human esophagus was cultured for 7 days prior to incubation with 100 μM [14C]-labeled N-nitrosamines (17) [DMNA (46 mCi/mmol), [methyl-14C]-EMNA (17.7 mCi/mmol), [1-ethyl-14C]-EMNA (19 mCi/mmol), [1-ethyl-14C]-DENA (14.5 mCi/mmol), [2,5,14C]-NPy (15.8 mCi/mmol), [methyl-14C]-DENA (4.59 mCi/mmol), N,N-[benzyl-14C]-BMNA (6.73 mCi/mmol), New England Nuclear, Boston, Mass.]. After incubation of rat esophagus explants with DMNA (precultured for 1 or 7 days), no significant difference in the level of radioactivity associated with DNA (424 and 372 dpm/μg DNA, respectively) was observed. Due to the difficulties of maintaining rat esophagus explant at sufficient size for biochemical experiments under rocking culture conditions, 1 day of culture prior to treatment with carcinogen was used for all the experiments with rat tissues.

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1 The abbreviations used are: BMNA, N-nitrosobenzylmethylamine; DMNA, N-nitrosodimethylamine; 7-MeGua, 7-methylguanine; NPy, N-nitrosopyrrolidine; EMNA, N-nitrosoethylnitrosamine; DENA, N-nitrosodimethylamine; 06-MeGua, O6-methylguanine; DNP, 2,4-dinitrophenylhydrazine.
For each experimental variable, 18 explants from rats and 6 explants from humans were placed in 3 sterile 60-mm plastic dishes (Falcon Plastics, Oxnard, Calif.), each containing 3 ml of medium. The dishes were then placed on a rack in a closed modified container [Nalgene plastic jar (500 ml)] (14), placed on a rocker platform, and rocked for approximately 5 cycles/min for 24 hr. To quantitate 14CO2 formed by metabolism of the N-nitrosamine the containers were flushed with N2 for 5 min, and the 14CO2 was trapped in 2 tubes, each containing 8 ml 0.2 M Ba(OH)2 as BaCO3. After removal of the explants, 1 ml 3 M phosphoric acid (pH 3) was added to each culture dish to release 14CO2 dissolved in the medium, and the container was flushed with N2 for another 5 min. The precipitate was collected on Whatman GF/C filters and washed with absolute ethanol until the radioactivity in the washing solution was negligible (<25 cpm). Media without explants of esophagus served as controls. The precipitate and filter were suspended in 3 ml water and 10 ml Aquasol liquid scintillation cocktail (New England Nuclear) and counted using internal standardization for efficiency calculations.

The mucosa was scraped from the explants, and DNA was isolated by phenol extraction and purified on a CaCl2 gradient. The binding value was determined as described previously (5). One explant from each experimental variable was fixed in 4% formaldehyde and 1% glutaraldehyde (pH 7.4) and prepared for light microscopy (7).

Carcinogen:DNA Adducts in Rat Esophagus

For identification of the DNA adducts formed by DMNA, DENA, [methyl-14C]BMNA, and [benzyl-14C]BMNA, the mucosa from 50 to 150 mg DNA was isolated as described previously (5). Mucosa from 50-150 mg DNA was isolated from 2,720 ± 270 (3) human esophagus could transform all the investigated N-nitrosamines, including DMNA, EMNA, DENA, NPY, and BMNA, human esophagus metabolically activate the different N-nitrosamines into DMA-, DENA-, BMNA-, and BMNA-, respectively.

Metabolites

Detection of other N-nitrosamines in Tissue Culture Media. The N-nitrosamines were concentrated by an extraction procedure. The media (1 to 2 ml) were poured over a small Preptube (Thermo Electron Corp., Waltham, Mass.) precoated with 5 ml dichloromethane. The sample was eluted with 5 x 5 ml dichloromethane and concentrated using a Kuderna-Danish apparatus. The analysis of volatile N-nitrosamines was performed by Thermo Electron Corp. on a Hewlett-Packard Co. gas chromatograph (Model 5710A; column, 0.25-inch x 6-foot glass, 10% Carbowax 20 M plus 0.5% KOH on Chromosorb, flux-calculated diatomite of low density 80 to 100 mesh) using a Thermoelectron Analyzer Model 50Z analyzer as detector.

RESULTS

The capability of cultured human and rat esophagus to metabolically activate the different N-nitrosamines into DNA-binding products was quantitated by analysis of radioactivity associated with DNA after incubation of explants with 14C-labeled substrates for 24 hr (Table 1). While cultured rat esophagus could transform all the investigated N-nitrosamines, DMNA, EMNA, DENA, NPY, and BMNA, human esophagus

<table>
<thead>
<tr>
<th>N-Nitrosamine</th>
<th>Radioactivity bound to DNA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td></td>
<td>dpm/mg DNA</td>
</tr>
<tr>
<td>DMNA</td>
<td>3,830 ± 735</td>
</tr>
<tr>
<td>[methyl-14C]EMNA</td>
<td>4,040 ± 670</td>
</tr>
<tr>
<td>[ethyl-14C]EMNA</td>
<td>680 ± 120</td>
</tr>
<tr>
<td>DENA</td>
<td>1,270 ± 99</td>
</tr>
<tr>
<td>[methyl-14C]BMNA</td>
<td>19,300 ± 3,480</td>
</tr>
<tr>
<td>[benzyl-14C]BMNA</td>
<td>300 ± 430</td>
</tr>
<tr>
<td>NPY</td>
<td>2,720 ± 270</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* Numbers in parentheses, number of individual experiments.
* Mean value.
* Range of values.
* ND, not detectable.
metabolized only the symmetrical N-nitrosamines, DMNA and DENA, to any significant degree. The highest binding in rat esophagus was seen with the unsymmetrical compounds, BMNA and EMNA. The major site of oxidation in BMNA was at the methylene group as shown by a higher level of methylation and by the formation of benzaldehyde. Methylation was observed at positions 7 and O6 of guanine (Table 2). The total binding level of BMNA, including both benzylidyne and methylation, was about 20-fold higher than that of EMNA. However, not enough radioactivity was associated with DNA after incubation with [benzyl-14C]BMNA to identify the alkylation products. After incubation of rat esophagus with [methyl-14C]EMNA, only 7-MeGua was detected, but the amount of radioactivity was too low to detect any O6-MeGua. The mean binding levels of the symmetrical N-nitrosamines, DMNA and DENA, were similar in human and rat esophagus. Rat esophagus was alkylated at positions 7 and O6 of guanine, but the major proportion of the radioactivity was associated with guanine and adenine (Table 2).

To investigate if any other N-nitrosamines were formed as intermediates in the metabolism of EMNA, DENA, or NPY, the culture media were extracted with methylene chloride, and the organic extracts were analyzed by gas chromatography using a TEA detector. No volatile A/-nitrosamine metabolites were detected by this analytical procedure.

The first step in the metabolic degradation of N-nitrosamine is the introduction of a hydroxyl group. When N-nitrosamines are hydroxylated in the α position, they become unstable and will spontaneously decompose under the formation of aldehyde, molecular nitrogen, and a carbonium ion. The aldehydes can be trapped by DNP under formation of hydrazone derivatives. These products can be separated by either thin-layer chromatography or high-pressure liquid chromatography. In rat esophagus, benzaldehyde (5.5 pmol/10 ml medium) was formed 10-fold higher than formaldehyde (0.6 pmol) from BMNA, another indication that the benzyl group is the major site for oxidation. Higher levels of acetaldehyde (0.08 pmol) were also formed from EMNA than formaldehyde (0.03 pmol). Three different aldehydes were formed from NPY when incubated with rat esophagus: formaldehyde (0.11 pmol); 4-hydroxybutanal (0.63 pmol); and an unknown (0.10 pmol) with a lower retention time than 4-hydroxybutanal. Only acetaldehyde was formed from DENA (0.10 pmol), and formaldehyde was formed from DMNA (2.47 pmol).

CO2 was detected as a metabolite from all the N-nitrosamines in rat esophagus (Table 3). The greatest amount was formed from DENA, a surprising observation since this compound is labeled at position 1. Higher levels of CO2 were also formed from the ethyl group than from the methyl group of EMNA.

**Table 2**

<table>
<thead>
<tr>
<th>BMNA (pmol)</th>
<th>DMNA (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial peak</td>
<td>7367 (43)*</td>
</tr>
<tr>
<td>Guanine</td>
<td>2190 (13)</td>
</tr>
<tr>
<td>Adenine</td>
<td>2248 (13)</td>
</tr>
<tr>
<td>7-MeGua</td>
<td>4545 (27)</td>
</tr>
<tr>
<td>O5-MeGua</td>
<td>546 (3)</td>
</tr>
<tr>
<td>3-methyladenine</td>
<td>73 (0.5)</td>
</tr>
<tr>
<td>O5-MeGua:7-MeGua ratio</td>
<td>0.120 0.302</td>
</tr>
</tbody>
</table>

* Number in parentheses, percentage of pmol.

**Table 3**

<table>
<thead>
<tr>
<th>N-Nitrosamine</th>
<th>14CO2 (pmol/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMNA</td>
<td>0.54 ± 0.14*</td>
</tr>
<tr>
<td>[methyl-14C]EMNA</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>[ethyl-14C]EMNA</td>
<td>1.69 ± 0.30</td>
</tr>
<tr>
<td>DENA</td>
<td>7.14 ± 0.25</td>
</tr>
<tr>
<td>[methyl-14C]BMNA</td>
<td>0.88 ± 0.48</td>
</tr>
<tr>
<td>[benzyl-14C]BMNA</td>
<td>0</td>
</tr>
<tr>
<td>NPY</td>
<td>1.14 ± 0.53</td>
</tr>
</tbody>
</table>

* Mean ± S.D. of 3 independent experiments.

**DISCUSSION**

N-nitrosamines are generally organotroop carcinoformers in experimental animals, and the organ selectivity is dependent on both the type of N-nitrosamine and the animal species (31). Thus far, this group of carcinogens has not been implicated in the etiology of human cancer, although they are found to be present in our environment. Since N-nitrosamines are procarcinogens, organotropism could partly be explained by the ability of the different organs to metabolize the N-nitrosamines into their ultimate carcinogenic form. α-Hydroxylation has been considered the most probable route of metabolic activation of N-nitrosamines to carcinogenic intermediates (29). However, most of the metabolism studies have been carried out with microsomal preparations rather than with intact cells from the target organ in which the carcinogenic response is observed. For example, α-hydroxylation of NPY is the only detectable oxidative pathway when liver or lung microsomes were incubated with NPY (17–20), whereas in whole animals, β-oxidation products of NPY were produced and excreted as urinary metabolites (9, 25). Therefore, it is important to identify the oxidative pathways of N-nitrosamine in organs that are susceptible and nonsusceptible to the neoplastic responses. Organ differences in the metabolism of N-nitrosobis(2-oxopropyl)amine have been shown in the hamster in vivo (27). In previous studies, we have shown that cultured human bronchus (14, 15), colon (2), and bladder cells (1) can activate both cyclic and acyclic N-nitrosamines into metabolites which reacted with DNA or were incorporated into DNA, while human esophagus activated only DMNA (16).

Several unsymmetrical N-nitrosamines such as BMNA selectivity induce esophageal cancer in experimental animals when given in drinking water. This study indicated that BMNA was more extensively metabolized than were the other N-nitrosines by cultured rat esophagus. The major site for oxidation of BMNA was the methylene group rather than at the methyl group. Oxidation of the methylene group results in the formation of benzaldehyde, which could be further oxidized, and of methyl carbonium ion, which reacted with DNA. Our result shows a 10-fold-higher binding level of the methyl group than of the benzyl group to DNA in rat esophagus. In parallel, benzaldehyde was formed in larger quantities than were formaldehyde and CO2. The major sites of DNA methylation by BMNA were positions O6 and 7 in guanine with the O6-MeGua:7-MeGua ratio similar to the ratio after in vivo administration of BMNA (21). However, the ratio is slightly higher than in rat liver after administration of DMNA (11). Both benzaldehyde and formaldehyde were formed by human esophaga-
Alklylation of rat esophageal DNA by BMNA has been shown previously in vivo after i.p. injection (11, 21) and in vitro when slices of rat esophagus were incubated with BMNA (11). In addition, BMNA was metabolized to a higher degree, as measured by binding to DNA, in the esophagus than in any other organs investigated (11). More benzaldehyde was also formed from BMNA when incubated with rat esophageal microsomes than were other microsomal preparations (35). Hippuric acid was the major metabolite excreted in the urine after treatment of rats with BMNA (23).

DMNA was predominantly oxidized at the ethyl group as measured by the high level of $^{14}$CO$_2$ and by a higher level of binding to DNA of the methyl group. Similar to our observations, oxidation by rat liver microsomes at the methylene group to acetaldelyde is faster than the oxidation to formaldehyde (8, 26). However, using deuterium-labeled EMINA, Lijinsky and Reuber (28) showed that an ethylating agent rather than a methylyating agent is involved in carcinogenesis by EMINA.

Oxidation of NPY occurs at the $\alpha$ position using either human or rat liver microsomes in vitro (17, 18, 20) and at the $\beta$ position in rat in vivo (9, 25), although CO$_2$ is the major metabolite in vivo (25, 36). It has been generally assumed that $\alpha$-oxidation of NPY is the most important pathway to produce a carcinogenic metabolite (18). In cultured rat esophagus, we have found evidence for $\alpha$-oxidation, as shown by the formation of 4-hydroxybutanal identified as the derivative of DNP. Although no alkylation of NPY metabolite to human esophageal DNA was detected, metabolism took place as detected by the formation of 4-hydroxybutanal and formaldehyde. Formation of formaldehyde and CO$_2$ could originate from $\beta$-oxidation followed by rearrangement under ring opening. However, none of the possible aldehyde:ketone formed by such rearrangement has been detected in the tissue culture media. Evidence for both $\alpha$- and $\beta$-oxidation of NPY in intact tissues has been found using human bladder cells, as shown by the formation of both 4-hydroxybutanal (1) and N-nitrosopyrrolidin-3-ol (33). The latter compound was identified by gas chromatography and mass spectrometry of the silylated product. The alkylation species formed from NPY is still unidentified. We have reported the presence of $[^{14}$C]methanal, detected as the ester of 3,5-dinitrobenzoic acid, in the media after incubating human colon with NPY (3), which indicates the formation of the methyl carbonate ion.

Aldehydes formed during the metabolism of N-nitrosamines may have important biological consequences. For example, formaldehyde had been shown recently to cause nasal cavity carcinomas in rats (39) and at the molecular level to form cross-links between cellular macromolecules (34). Since these aldehydes are produced (a) following hydroxylations of the parent N-nitrosamine and (b) in equilibrium concentrations and at the same intracellular sites as the alkylyating species, the possible interactive effects of aldehydes and alkylyating agents in both carcinogenesis and the organotropism of N-nitrosamines should be investigated.

The metabolism of DENA and DMNA as measured by the alkylation of DNA was similar in both rat and human esophagus. In cultured rat esophagus, a significantly greater amount of CO$_2$ was formed following the oxidation of DENA than that produced from DMNA; similar results have been obtained with tissue slices of several rat extrahepatic tissues (32). While most of the radioactivity associated with DNA in rat esophagus was associated with the incorporation of carbon 1 fragments into DNA bases, both O$^3$-MeGua and 7-MeGua were detected by liquid chromatography. The O$^3$-MeGua:7-MeGua ratio was higher than that observed in cultured human esophagus (16) and after incubation of rat esophagus with [methyl-$^{14}$C]BMNA.

Metabolism of N-nitrosamines is an essential step for these compounds to exert their carcinogenic effect. One end point for the metabolism is the binding of metabolites to cellular macromolecules including DNA, a quantitation which, however, does not correlate with carcinogenic potential. Identification of the carcinogen-DNA modifications is necessary before any such correlation can be made. Quantitation of the different adducts in cultured tissue is difficult due to limitations in the sensitivity of the assay due to low specific activity of the radioactive-labeled N-nitrosamines. However, it is interesting that rat esophagus, which is a major target organ for N-nitrosamine carcinogenesis, could activate all the N-nitrosamines that we have tested, while human esophagus could activate only the symmetrical acyclic compounds to metabolites that bind to DNA. However, the use of slightly different culture conditions for human and rat esophagus could have an effect on these results, although preliminary experiments did not indicate any quantitative differences with different preculture periods prior to carcinogen treatment. This is the first example in our explant culture model systems of a possible qualitative difference in the ability of an organ from humans and animals to metabolize carcinogens, although wide quantitative differences have been observed (4, 5).

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