Methylation versus Ethylation of DNA in Target and Nontarget Tissues of Fischer 344 Rats Treated with N-Nitrosomethylethylamine


ABSTRACT

Bioactivation of N-nitrosomethylethylamine can be initiated by hydroxylation of either the methyl or ethyl moiety leading to an ethylating or methylating intermediate, respectively. This study was designed to determine which of these metabolic pathways predominates in vivo and to what extent DNA is alkylated in the target and nontarget tissues. Adult male Fischer 344 rats received a single i.p. or p.o. dose (4.4 mg/kg, 0.05 mmol/kg) of N-nitrosomethylethylamine, 14C-labeled in either the methyl or ethyl group (survival time, 4 h). DNA was analyzed by Sephasorb-HP chromatography following acid hydrolysis in 0.1 M HCl. Concentrations of 7-methylguanine in hepatic DNA were 170-200 times higher than those of 7-ethylguanine. This is approximately 2.6 times the 7-methylguanine:7-ethylguanine ratio of 68, observed when DNA is reacted in vitro with equimolar amounts of the direct alkylating agents N-nitrosomethylurea and N-nitrosoethyurea, suggesting that hydroxylation at the α-position of the ethyl group of N-nitrosomethylethylamine proceeds at about 2.6 times the rate as at the methyl group. Concentrations of 7-methylguanine in liver were approximately 15 times higher than in kidney, 100 times higher than in esophagus, and 200 times higher than in lung. Addition of ethanol to the drinking water (5%) caused a slight interorgan shift in metabolism with a decrease in the 7-methylguanine ratio for liver:esophagus by 50% and an increase in the 7-methylguanine ratio for liver:kidney by 40%.

INTRODUCTION

NMEA displays carcinogenic properties other than expected from the activity of structurally related nitrosamines. In rats it is a weaker carcinogen than either N-nitrosodimethylamine or N-nitrosodiethylamine (1,3) and does not follow the general principle established by Druckrey et al. (1) that asymmetric nitrosamines produce predominantly esophageal tumors. When administered chronically to adult Fischer 344 rats in drinking water (30 ppm), NMEA induces hepatocellular carcinomas in more than 45% of animals treated (3). At higher doses (150 ppm), hemangiosarcomas (85%) and esophageal neoplasms (35%) were also observed (2) in addition to hepatocellular carcinomas (95%). As in the case of other dialkynitrosamines (1), hydroxylation at one of the α-carbon atoms is assumed to represent the initial step in the bioactivation of NMEA. Hydroxylation of the methyl group would yield formaldehyde and ethyl diazonium ion as the ultimate carcinogen. A methylating intermediate would result from hydroxylation at the α-carbon position of the ethyl group, thereby producing acetaldehyde and methyl diazonium ion. In vitro studies using hepatic microsomes from adult rats showed the production of formaldehyde and acetaldehyde, indicating that both metabolic pathways occur (4,5). It was further demonstrated that there is a clear binding of radioactivity from [1-ethyl-14C]NMEA to calf thymus DNA in a rat liver microsome catalyzed system (6). However, no data are available on the relative extent of DNA methylation versus ethylation in vitro or in the intact animal, which are useful in evaluating the relative importance of methylation and ethylation of DNA in malignant transformation. In the present study, adult male Fischer 344 rats were given NMEA 14C-labeled in the methyl or ethyl position. Radiochromatographic DNA analyses indicate that the metabolism of NMEA in rat liver occurs predominantly at the α-carbon of the ethyl group leading preferentially to a methylation of hepatic DNA.

MATERIALS AND METHODS

Animals. Young adult male Fischer 344 rats (120–150 g body weight; Charles River, Wiga, Federal Republic of Germany) were maintained on a standard laboratory diet and given water ad libitum.

Chemicals. [1-Ethyl-14C]NMEA was prepared as follows. An ethanolic solution of sodium-1-[14C]acetate (30 mCi, 50 mCi/mmol) was evaporated and mixed with 56 mg of vacuum-dried methylamine hydrochloride. The mixture was dissolved in 3 ml of liquid sulfur dioxide and reevaporated. The residue was stirred with 190 mg of dicyclohexylcarbodiimide in dry tetrahydrofuran (1 ml) in a capped vial for 48 h, after which excess coupling agent was destroyed by the addition of 12 mg of methanol and 23 mg of acetic acid in 1 ml of tetrahydrofuran. After stirring for 1 h, the solution of crude N-methyl[1-14C]acetamide was filtered, diluted with 10 ml of dry tetrahydrofuran, treated with 300 mg of lithium aluminum hydride, and stirred for 4 days at room temperature. Excess reductant was destroyed by cautious addition of aqueous tetrahydrofuran, and the solids were dissolved by dropwise addition of concentrated sodium hydroxide solution. The reaction mixture was distilled to near-dryness, with noncondensed gases being delivered into 5 ml of 2 M hydrochloric acid. Two additional 5-ml portions of water were added, with each being followed by distillation to near-dryness. The distillate and acid solution were combined and concentrated in a stream of nitrogen to remove tetrahydrofuran. The remaining solution containing the [1-ethyl-14C]methylamine hydrochloride was cooled in ice, mixed with 5 ml of acetic acid and 2 g of sodium nitrite, stirred at room temperature overnight, basified with concentrated sodium hydroxide, and extracted 5 times with a total of 20 ml of dichloromethane. The combined extracts were filtered into a Kuderna-Danov evaporator and covered with 0.5 ml of water. The organic solvent was slowly evaporated at a bath temperature of 60°C. The remaining aqueous solution was injected onto a preparative Vydac...
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C-18 high-pressure liquid chromatography column (3.9 x 300 mm, 10 μm) and eluted with 0.01 M phosphate buffer (pH 7.3) at a flow rate of 1.2 ml/min. The nitrosamine reached its maximum concentration in the eluate 13 min after injection. It was collected in fractions of 6.5 and 7.1 μm; the concentrations were estimated from their absorbances at 332 nm to be 0.71 and 0.32 mg/ml, respectively. The overall yield based on acetate was 10%. Specific activity was 50 mCi/mmol; unlabeled NMEA was added to give a specific activity of 13.5 mCi/mmol. The nitrosamine was radiochromatographically pure by gas chromatography (10% Carbowax, 20 μm column at 80°C, gas proportional counter) and high-pressure liquid chromatography (C₆H₅Bondapak column, 0.01 m phosphate buffer as eluent). By thin-layer chromatography, the radiochemical purity was 97%, with 3% of the recovered cpm at the origin. Unlabeled NMEA was prepared for reference purposes by conversion of cold sodium acetate in 30% isolated yield using the above procedure. Its mass spectrum was identical to that of an authentic standard.

[Methyl-¹⁴C]NMEA (19 mCi/mmol) was obtained from Amersham International and used at a specific activity of 4.5 mCi/mmol. The radiochemical purity of the methyl- and ethyl-labeled compounds was analyzed immediately before use by high-pressure liquid chromatography on Lichrosorb RP-8 columns (4.6 x 150 mm), eluted with 5% aqueous methanol (v/v), and found to be greater than 95% and 98%, respectively. Sephasorb-HP was purchased from Pharmacia Fine Chemicals, Sweden. Kontroigel and Riatron scintillation cocktails were purchased from Kontro AG, Zürich, Switzerland. DNA-grade hydroxylapatite was from Bio-Rad Laboratories AG, Glatbrugg, Switzerland. All other chemicals were of analytical grade purity or higher.

Metabolic Cage. Two rats received a single i.p. dose of [methyl-¹⁴C]-NMEA (0.44 or 44.0 mg/kg; 2.5 and 0.022 mCi/mmol, respectively) and were monitored for expired ¹⁴CO₂ in a metabolic cage (Jencsons Metabo-2, Hennel Hempstead, United Kingdom). Samples (0.5 ml) were collected from two serially connected Nilox columns (each containing 600 ml of 1 N NaOH) and counted for ¹⁴C after the addition of 1.0 ml H₂O and 10 ml Kontroigel (87% counting efficiency).

DNA Methylation and Ethylation by NMEA in Vivo. Rats were given [methyl-¹⁴C]NMEA (4.4 mg/kg; 4.5 mCi/mmol) or [ethyl-¹⁴C]NMEA (4.4 mg/kg; 13.5 mCi/mmol) i.p. or p.o. in the drinking water (4.0 ml) with or without 5% ethanol (v/v). Animals receiving NMEA in the drinking water were deprived of water for 18 h prior to the experiment. Groups of 6 rats were used for the determination of methylated bases, while groups of 3 rats were used for the determination of ethylated bases. Animals were killed 4 h after administration of radiolabeled NMEA. Esophagus, liver, kidney, and lungs were rapidly removed, frozen in liquid N₂, and stored at −70°C.

DNA isolation. DNA was isolated from 2–4 g of tissue using a modification of the hydroxylapatite method (7, 8). Tissues were allowed to thaw at 4°C in 10 ml of 1% sodium laurylsulfate per g of tissue (in Tris:EDTA:NaCl, 10 mM each, pH 8.0) and homogenized (Potter Elvejem). The homogenate was incubated with proteinase K (0.5 mg/ml homogenate) at 37°C for 30 min. An equal volume of urea:buffer (8 M urea; 0.14 M sodium phosphate, pH 6.8) was added, and the homogenate was incubated for another 30 min at 37°C. Proteins were extracted with 10 ml of chloroform:isoamyl alcohol:phenol (24:1:25, v/v/v) per g of tissue by agitation at room temperature for 15 min. The organic and aqueous phases were separated by centrifugation at 2000 × g for 10 min (4°C). The aqueous supernatant was reextracted with one-half its volume in chloroform:isoamyl alcohol:phenol. The supernatant was then added to 3 g of hydroxylapatite per g of initial tissue weight which had been previously conditioned by heating in 10 ml of urea:buffer to 85°C for 10 min. After agitation at room temperature for 30 min, the slurry was centrifuged at 1500 × g, and the supernatant was decanted. The hydroxylapatite was washed twice with 10 ml of urea:buffer (for 3 g of hydroxylapatite) and twice with 10 ml of 0.1 M phosphate buffer (pH 6.8). DNA was removed from the hydroxylapatite by 3 washings with 10 ml of 0.5 M phosphate buffer (pH 6.8). The combined supernatants were centrifuged at 1500 × g to remove traces of hydroxylapatite. To precipitate DNA, 100 μl of 0.5 M EDTA:Tris (pH 6.0) and 20 μl of 5% cetylpyridinium bromide were added per ml of supernatant (9). This mixture was then kept at −20°C overnight. After thawing, DNA was centrifuged at 3000 × g for 10 min (20°C). The precipitated DNA was washed with 30 ml of ice-cold water and recentrifuged at 3000 × g for 10 min. Five ml of 0.1 M sodium acetate in 80% ethanol (pH 4.5) were then added, followed by 15 ml of absolute ethanol. The DNA was again pelleted by centrifugation at 1750 × g for 30 min at −10°C. It was then washed once with absolute ethanol and once with ether and allowed to dry at room temperature. DNA was stored at −70°C until use.

Radiochromatography of DNA Hydrolysates. Immediately prior to analysis, DNA was depurinated in 0.1 M HCl at 80°C for 30 min and subsequently filtered through a 0.45-μm Gelman ACRO LC13 filter. Purine bases were separated on Sephasorb-HP columns (1 x 50 cm) as previously described (10). Samples were eluted with 10 ml phosphate buffer (pH 5.5) at a flow rate of 1.4 ml/min (fraction volume, 3.65 ml). The eluent was monitored for absorbance at 254 nm using an LKB Uliv-cord SD detector coupled with a Shimadzu C-R1B integrator. Absorbance was additionally determined in individual fractions at 260 nm using a Shimadzu UV-240 spectrophotometer, so that fractions containing high amounts of unmodified bases could be diluted for more accurate quantification. Radioactivity was determined after the addition of 6.0 ml of scintillation cocktail (Riatron, 80% counting efficiency). Amounts of alkylated purines were expressed as μmol per mol of guanine assuming that their specific activity was the same as that of the injected nitrosamine.

RESULTS

The rate of metabolism of NMEA as determined by monitoring exhaled ¹⁴CO₂ after i.p. administration of 44.0 and 0.44 mg of [methyl-¹⁴C]NMEA per kg is shown in Fig. 1. At the lower dose, the production of ¹⁴CO₂ reached a plateau after approximately 3 h, and at the higher dose, after about 9 h. The times at which half the maximal amount of ¹⁴CO₂ was exhaled (t½ max) were 1.5 h and 3.8 h, respectively. Of the total amount of [methyl-¹⁴C]-NMEA administered, 60–65% was exhaled as ¹⁴CO₂.

Representative Sephasorb-HP chromatograms of hydrolyzed hepatic DNA from rats treated with NMEA ¹⁴C-labeled in either the methyl or ethyl position are shown in Fig. 2. Hepatic DNA from animals receiving [methyl-¹⁴C]NMEA contained 7-methylguanine and O₆-methylguanine as the major alkylated purines.

![Fig. 1. Metabolism of [methyl-¹⁴C]NMEA following a single i.p. dose of 0.44 mg/kg (⊗) and 44.0 mg/kg (△). Exhaled ¹⁴CO₂ is expressed as a cumulative percentage of the total amount of [methyl-¹⁴C]NMEA injected.](image-url)
Metabolic incorporation into guanine and adenine was minimal. As expected from the findings of other investigators (11), ethylated bases had a somewhat longer retention time than did their methyl analogues. DNA from rats treated with [1-ethyl-14C]NMEA contained in addition to 7-ethylguanine and O-ethylguanine an early peak (Fig. 2, Fractions 11--14) which was not positively identified. Its retention time was similar to that of O-methylguanine and 7-(2-hydroxyethyl)guanine, but the amount of radioactivity present was insufficient to resolve this peak on a different chromatographic system. Similarly, a radioactive peak suspected to represent O-ethylguanine (bottom). DNA isolation and Sephasorb-HP chromatography were as described in the text. Inset, O-ethylguanine (O-ethylG) peak on an expanded scale. O-ethylG, O-ethylguanine.; extinction; • •, dpm.

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Fig. 2. Chromatographic profiles of hepatic DNA hydrolysates from rats which received a single i.p. injection (4.4 mg/kg) of [methyl-14C]NMEA (top) or [1-ethyl-14C]NMEA (bottom). DNA isolation and Sephasorb-HP chromatography were as described in the text. Inset, O-ethylguanine (O-ethylG) peak on an expanded scale. O-ethylG, O-ethylguanine.; extinction; • •, dpm.

DISCUSSION

The present study clearly shows that NMEA gives rise to both methylation and ethylation of cellular DNA in vivo, indicating that \(\alpha\)-carbon hydroxylation occurs at both the methyl and ethyl group of NMEA. In rat liver DNA, the levels of 7-methylguanine were 170--200 times higher than those of 7-ethylguanine. This ratio does not, however, directly reflect the rate of metabolism of NMEA to a methylating or ethylating intermediate, since both the extent of alkylation and the pattern of alkylation products differ considerably for methylating and ethylating nitrosamines. Specifically, when equimolar concentrations (20 mM) of the direct acting N-nitroso compounds N-nitrosomethylurea and N-nitrosoethyurea were incubated with DNA in vitro, the overall extent of alkylation (mmol of alkyl group per mol of DNA:phosphate) by N-nitrosomethylurea is 12 times higher than for 

4L Keefer, unpublished data.

While the biological basis of the organ-specific carcinogenicity of \(N\)-nitroso compounds is not yet fully understood, the overall extent of reaction with DNA and the capacity of different tissues to enzymically repair promutagenic O-alkylated DNA bases have
emerged as likely key factors (13, 21). In particular, there is evidence that O\(^6\)-alkyl guanine and O\(^4\)-alkyl thymine may cause stable mutations following mispairing during DNA replication (22). The O\(^6\)-methylguanine:7-methylguanine ratio observed in rat liver (Table 1) was close to the "theoretical" value of 0.11 observed after reaction of methylating nitroso compounds with DNA in vitro, indicating that repair of O\(^6\)-methylguanine had not occurred to any significant extent. This can be explained by the fact that the O\(^6\)-alkylguanine:DNA alkyltransferase is saturated in rat liver at concentrations of O\(^6\)-methylguanine above 184 \(\mu\)mol/mol of guanine (16, 17). The O\(^6\)-ethylyguanine:7-ethylguanine ratio in liver averaged 0.58 ± 0.07 (Table 1), which is again close to the in vitro ratio of 0.68-0.71 (18, 23). In kidney DNA, the O\(^6\)-methylguanine:7-methylguanine ratio was approximately 0.05, indicating that in this organ some repair had taken place during the experiment.

In rat liver, the principal target tissue for NMEA, 7-methylguanine concentrations were approximately 15 times higher than in kidney, 100 times higher than in esophagus, and more than 200 times higher than in lung (Table 1). From these data, one would expect that the kidney would be the most likely extrahepatic target tissue for malignant transformation by NMEA. This organ does, in fact, develop tumors after a single high dose of methylating nitrosamines (e.g., N-nitrosomethylamin; Ref. 24) but not after chronic administration (25). NMEA has to date only been tested by chronic administration. The extent of esophageal DNA alkylation by NMEA was found to be very low, but the results of chronic administration indicate that this is sufficient to produce esophageal neoplasms (including papillomas) in 35% of exposed animals (2). In this respect NMEA shares properties with N-nitrosodimethylamine which also produces esophageal tumors (1, 2). N-Nitrosodimethylamine, in contrast, does not produce esophageal tumors at any dose or route of application and does not methylate esophageal DNA to any measurable extent.

Ethanol has been demonstrated to markedly affect the metabolism of some nitrosamines (26). When administered simultaneously with N-nitrosomethylbenzylamine, ethanol causes a marked inhibition of hepatic metabolism, leading to increased exposure of extrahepatic tissues (27), with a 2.5-fold increase in methylation of esophageal DNA and a 5-fold increase in methylation of DNA in lung. Administration of NMEA in H\(_2\)O containing 5% ethanol p.o. also caused an interorgan shift in the metabolism of NMEA (Table 1). The liver:esophagus ratio of 7-methylguanine decreased by 50%, indicating a shift in metabolism towards the esophagus. The absolute extent of DNA methylation in hepatic DNA was reduced by approximately 13%, and in renal DNA by 47%. Since decreased \(\alpha\)-hydroxylation of the ethyl group of NMEA would lead to decreased methylation, this is in accordance with observations by Swann et al. (28) who demonstrated that ethanol inhibits DNA ethylation by N-nitrosodimethylamine in both liver and kidney. Methylation of kidney DNA by N-nitrosodimethylamine, in contrast, is increased by concurrent ethanol administration (26). This suggests that \(\alpha\)-hydroxylation of methyl and ethyl groups is mediated by isozymes of the microsomal cytochrome P-450 system (29) differing in their susceptibility to inhibition by ethanol. The preferential inhibition by ethanol of \(\alpha\)-hydroxylation of the ethyl group of NMEA would explain why there was proportionately more ethylation of kidney DNA in animals receiving a p.o. dose of NMEA concurrently with ethanol (Table 1). This carries the implication that ethanol may cause not only interorgan shifts in DNA alkylation (21, 26, 28) but also changes in the pattern of adducts formed by asymmetric dialkyl nitrosamines.

### Table 1

DNA alkylation by N-nitrosomethylamine in vivo

<table>
<thead>
<tr>
<th>Organ</th>
<th>Alkylpurine</th>
<th>([\text{methyl}^{14}\text{C}]\text{NMEA})</th>
<th>([\text{ethyl}^{14}\text{C}]\text{NMEA})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>(7)-Alkylguanine</td>
<td>1014 ± 7(^a)</td>
<td>60 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(O^6)-Alkylguanine</td>
<td>103 ± 3.5</td>
<td>0.5 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>(O^6/-7)-Alkylguanine</td>
<td>0.10 ± 0.01</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>7-Methylguanine/7-ethylguanine</td>
<td>29</td>
<td>40</td>
</tr>
<tr>
<td>Kidney</td>
<td>(7)-Alkylguanine</td>
<td>73(^d)</td>
<td>~0.4(^c)</td>
</tr>
<tr>
<td></td>
<td>(O^6)-Alkylguanine</td>
<td>4.0 (\text{ND}^d)</td>
<td>2.7(^c)</td>
</tr>
<tr>
<td></td>
<td>(O^6/-7)-Alkylguanine</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>7-Methylguanine/7-ethylguanine</td>
<td>~182</td>
<td>~60</td>
</tr>
<tr>
<td>Esophagus</td>
<td>(7)-Alkylguanine</td>
<td>9.3</td>
<td>ND</td>
</tr>
<tr>
<td>Lung</td>
<td>(7)-Alkylguanine</td>
<td>3.8(^b)</td>
<td>~0.3(^c)</td>
</tr>
<tr>
<td></td>
<td>7-Methylguanine/7-ethylguanine</td>
<td>~13</td>
<td>10</td>
</tr>
<tr>
<td>Liver/kidney ratio</td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Liver/esophagus</td>
<td>109</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Liver/lung</td>
<td>267</td>
<td>227</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD of 3 DNA samples isolated from pooled livers.

\(^b\) Mean of 2 DNA samples isolated from pooled tissues.

\(^c\) Single determination of DNA isolated from pooled tissues.

\(^d\) ND, not detectable; ~ indicates values for which the peak height (in cpm) was less than twice background.

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