Effects of ethanol and various alcoholic beverages on the formation of $O^6$-methyldeoxyguanosine from concurrently administered N-nitrosomethylbenzylamine in rats: a dose—response study

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Consumption of alcoholic beverages has been identified as a major cause of oesophageal cancer in industrialized countries, with an exceptionally high risk associated with apple-based liquors (calvados). In the present study, we have determined the dose—activity relationship of the effects of coincident ethanol on the formation of $O^6$-methyldeoxyguanosine ($O^6$-MdG) by the oesophageal carcinogen $N$-nitrosomethylbenzylamine (NMBzA). Male Fischer 344 rats received a single intragastric dose of NMBzA (2.5 mg/kg body wt; 7.4 ml/kg body wt) in tap water containing 0—20% ethanol (v/v). Survival time was 3 h. In controls, concentrations of $O^6$-MdG were similar in oesophagus, lung and liver (11—14.9 μmol/mol dG). In oesophagus, coincident ethanol increased levels of $O^6$-MdG from 15.2 μmol/mol (0.1% ethanol) to 46.0 μmol/mol (20%). This increase was dose dependent for 1—20% ethanol; however, low doses produced a larger effect per gram of ethanol than higher doses. In lung, concentrations of $O^6$-MdG increased from 11 μmol/mol (0.1%) to a plateau value of 24 μmol/mol (≥5%). In nasal mucosa, an increase in $O^6$-MdG from 3.9 μmol/mol (controls) to 30.7 μmol/mol was observed with 4% ethanol. Effects of ethanol on hepatic DNA methylation were statistically non-significant. Modulation of NMBzA bioactivation by various alcoholic beverages (adjusted to 4% ethanol) was also investigated. Increases in oesophageal $O^6$-MdG were similar (+50% to +116%) with pear brandy, rum, aquavit, vodka, brandy, gin, Scotch whisky, white wine, Pilsner beer and aqueous ethanol. Significantly higher increases were elicited by commercially distilled calvados (+125%) and red burgundy (+162%). In contrast to its effects at an ethanol content of 4%, farm-made calvados diluted to 20% ethanol produced significantly higher (+200%) increases in oesophageal DNA methylation than aqueous ethanol (+148%). Our results show that ethanol is an effective modulator of nitrosamine bioactivation in vivo at intake levels equivalent to moderate social drinking, and that some alcoholic beverages contain congeners that amplify the effects of ethanol, suggesting that modulation of nitrosamine metabolism by acute ethanol may play a role in the etiology of human cancer.

*Abbreviations: $O^6$-MdG, $O^6$-methyldeoxyguanosine; NMBzA, $N$-nitrosomethylbenzylamine.

Introduction

Consumption of alcoholic beverages and use of tobacco products are among the major causes of oesophageal cancer in industrialized countries (1). Epidemiological studies have demonstrated that though the effects of ethanol and tobacco are multiplicative (2,3), the relative risk for oesophageal neoplasms also increases progressively with daily intake of ethanol in nonsmokers (2,4,5). Moreover, the risk for tumour development is dependent on the type of alcoholic beverage consumed and appears to be more pronounced for distilled spirits than for beer or wine (1,2,6). An exceptionally high risk has been inferred for cider and apple brandy (calvados), which are traditionally popular beverages in the French provinces of Brittany and Normandy where mortality rates for oesophageal cancer in males are elevated by 3— to 4-fold above the European average (1,2,7). While neither ethanol nor alcoholic beverages are carcinogenic per se (8—10), dietary ethanol has been observed to alter the organotropism and tumorigenic potency of a number of carcinogenic nitrosamines in laboratory rodents (9,11—16). Several studies have demonstrated that moderate amounts of concurrently administered ethanol inhibit hepatic nitrosamine metabolism, resulting in increased nitrosamine bioactivation in extrapleural tissues in which monooxygenase activity either is not or is less sensitive to inhibition by ethanol (17—20). Nitrosamines, which are ubiquitous as environmental contaminants and as products of endogenous synthesis from ingested precursors, may constitute a substantial proportion of the total human carcinogen burden (21). An ethanol-mediated inter-organ shift in nitrosamine bioactivation could, therefore, significantly contribute to the enhancement associated with alcohol consumption of tumorigenesis in oesophagus and other organs in which malignant transformation in humans is otherwise less frequent (18).

In the present study, we have investigated the dose—activity relationship of modulation by ethanol of nitrosamine bioactivation. The effects of a single simultaneously administered dose of ethanol on the formation of the promutagenic base $O^6$-methyldeoxyguanosine ($O^6$-MdG*) from N-nitrosomethylbenzylamine (NMBzA), a highly potent and selective oesophageal carcinogen (22), were assessed in oesophagus, lung and liver over a wide range of ethanol concentrations. In addition, we examined whether alcoholic beverages, including commercially distilled and farm-made calvados, exert a shift in NMBzA bioactivation which exceeds that of a similar dose of ethanol alone.

Materials and methods

Chemicals and antibodies

RNase T1 from Aspergillus oryzae was purchased from Boehringer-Mannheim (Schweiz) AG, Rochezur, Switzerland. RNase A from bovine pancreas and calf thymus DNA was purchased from Sigma Chemie, D-8024 Deisenhofen, Germany. DNA purification-grade lysin buffer and 70% phenol/water/chloroform reagents were from Applied Biosystems, Inc., Foster City, CA, USA. Characteristics of the rabbit antiserum raised against keyhole limpet hemocyanin conjugates of $O^6$-methylguanosine (NPZ 193—1) have been described previously.
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(23) Goat anti-rabbit IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolylphosphate toluidine salt and nitroblue tetrazolium chloride were obtained from BioRad, Glattbrugg, Switzerland. All commercial chemicals were of analytical grade or higher.

Alcoholic beverages
Farm-made calvados was kindly provided by Dr. M. Mandard, Centre Francais-Bacless, Caen, France. All other alcoholic beverages were from commercial sources. Ethanol concentrations of the undiluted beverages (Table I) and of all diluting solutions were determined with a kit (cat. no. 176290) purchased from Boehringer-Mannheim (Schweiz) according to the manufacturer’s directions.

Animals and treatment
Male Fischer 344 rats (100–160 g) were obtained from Charles River Wiga GmbH, Kaiserslautern, Germany and maintained on a standard laboratory diet with water ad libitum. NMBzA was administered as a single dose of 2.5 mg/kg (18 μmol/kg) by gavage in a volume of 7.4 ml/kg body wt. For the determination of the dose–activity relationship of the effect of ethanol on DNA methylation by NMBzA, the carcinogen was dissolved in tap water containing 0, 0.1, 0.5, 1.5, 10 or 20% ethanol (v/v). Five animals were treated with each ethanol concentration. In a second experiment, groups of five animals received NMBzA dissolved in water, 4% aqueous ethanol, or one of nine alcoholic beverages (Table I). The ethanol content of the beverages was adjusted to a final concentration of 4% with tap water prior to adding the nitrosamine. After a survival time of 3 h, the animals were killed by exsanguination during ether anaesthesia. Tissues were removed rapidly, frozen in liquid nitrogen and stored at −70°C until analysis.

DNA isolation
DNA was isolated by automated phenolic extraction using a Model 340A Nucleic Acid Extractor (Applied Biosystems, Inc.). Liver tissue (0.3–0.5 g) was pulverized in liquid nitrogen, homogenized in 10 ml of PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM NaHPO₄, 1.5 mM KH₂PO₄, pH 7.2) with 10–12 passages in a Potter–Elvehjem homogenizer and filtered through a single layer of nylon gauze (30 μm mesh; Willi Fischer Labortechnik, Frankfurt/Main, Germany). Cell nuclei were collected by centrifugation for 5 min at 1000 g, suspended in 0.2–0.4 ml of PBS and transferred to 5 ml Potter–Elvehjem homogenization vessels. Following the addition of 2 ml of lysis buffer, the mixtures were homogenized by hand (three passages) and transferred to 3 ml of lysis buffer. Crushed oesophagi and nasal cavity scrapings (0.3 g) were homogenized directly in 5 ml of lysis buffer and filtered through nylon gauze. The crude nuclei or tissue homogenates were pre-digested with RNase A and T₂ (400 U/g tissue of each) at 37°C for 1 h. After adding proteinase K (85–170 U/g tissue), digestion was continued for an additional 2 h at 37°C and overnight at 4°C. Samples were transferred to the 14 ml vessels of the DNA extractor and extracted twice with 200 μl of 4 M ammonium acetate. An alkaline modification: DNA (12 μg in 200 μl) was denatured for 10 min at room temperature with 200 μl of 100 mM NaOH, neutralized with 200 μl of 15% (v/v) acetic acid and mixed with 200 μl of 4 M ammonium acetate. An alkaline phosphatase conjugated goat-anti-rabbit IgG antibody was used as a second antibody. Bound antibodies were visualized with 5-bromo-4-chloro-3-indolylphosphate toluidine salt (0.136 mg/ml) and nitroblue tetrazolium chloride (0.33 mg/ml) in diethanolamine buffer (0.1 M Tris–HCl, 0.1 M NaCl, 25 mM diethanolamine, 2 mM MgCl₂, 1 mM ZnCl₂, pH 9.55) (25). Densitometric evaluation was performed at 530 nm using a Shimadzu Model CS-930 dual wavelength thin-layer chromatogram scanner in the zig-zag mode. Standard curves were generated with DNA which was methylated in vitro with methyltransferase and calibrated by HPLC with fluorescence detection (26). Peak heights were corrected for background binding to unmodified DNA and plotted against C^3-MedG concentrations in a double log plot. The limit of quantitation was 1.5 μmol C^3-MedG/mol deoxyguanosine.

Statistical analysis
Mean and standard deviations of duplicate determinations in animals assessed individually were established by one-way analysis of variance. The double-tailed Student’s t-test was used to compare DNA methylation in rats receiving different alcoholic beverages. P values of 0.05 or less were considered significant.

Results
The effects of a single oral dose of ethanol on the bioactivation of simultaneously administered NMBzA in oesophagus, lung and liver are shown in Figure 1. Animals received a single oral dose of NMBzA (2.5 mg/kg; 7.4 ml/kg) which was dissolved in tap water containing ethanol at concentrations ranging from 0 to 20% (v/v), i.e. the total amount of ethanol administered was 0–1.2 g/kg. Levels of C^3-MedG were determined after a survival time of 3 h. In oesophagus, concentrations of C^3-MedG increased from 15 μmol C^3-MedG/mol dG in rats treated with 0.1% ethanol to 46 μmol/mol in animals given 20% ethanol. As shown in Figure 2, the log of C^3-MedG concentrations in this tissue increased linearly with the log of dose for solutions containing 1–20% ethanol. The regression coefficient, calculated by the least-squares method, was 0.31 (r = 0.999), indicating that the relative increase in formation of C^3-MedG in oesophageal DNA with respect to the amount of ethanol ingested was proportionally smaller at high doses. A dose-dependent increase in DNA methylation with increasing doses of ethanol was also observed in lung. In contrast to oesophagus, saturation at a plateau value of ~25 μmol/mol was achieved with 5% ethanol; the half-maximal effect was estimated to have been attained with 2.5% ethanol. In liver there was a marginal but statistically nonsignificant trend towards inhibition of DNA methylation with

Table I. Ethanol content of the alcohol beverages examined in this study.

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Ethanol (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilsner beer</td>
<td>39.8</td>
</tr>
<tr>
<td>White wine</td>
<td>10.2</td>
</tr>
<tr>
<td>Red burgundy wine</td>
<td>106.8</td>
</tr>
<tr>
<td>Sake</td>
<td>150.7</td>
</tr>
<tr>
<td>Pear brandy</td>
<td>329.8</td>
</tr>
<tr>
<td>Gin (Great Britain)</td>
<td>333.2</td>
</tr>
<tr>
<td>Commercially distilled calvados</td>
<td>333.2</td>
</tr>
<tr>
<td>Scotch whisky</td>
<td>353.6</td>
</tr>
<tr>
<td>Farm-made calvados</td>
<td>569.7</td>
</tr>
</tbody>
</table>

![Fig. 1. Influence of concurrently administered ethanol on DNA methylation by NMBzA in rat oesophagus, lung and liver. Concentrations of C^3-MedG were determined 3 h after a single dose by gavage of NMBzA (2.5 mg/kg body wt; 7.4 ml/kg) in tap water containing 0–20% (v/v) ethanol. Data represent mean ± SD of 4–6 determinations in pooled DNA from five animals.](https://academic.oup.com/carcin/article-abstract/13/7/1171/305777)
Effects of ethanol on O^-MEdG formation

Fig. 2. Dose-response curve for the increase in oesophageal DNA methylation by NMBzA elicited by simultaneously administered ethanol. Data are taken from Figure 1.

Table II. Effect of various alcoholic beverages on the formation of O^-MEdG from NMBzA in rat oesophagus

<table>
<thead>
<tr>
<th>Beverage</th>
<th>O^-MEdG/dG</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.8 ± 1.7p</td>
<td>0</td>
</tr>
<tr>
<td>4% ethanol (v/v)</td>
<td>25.8 ± 3.8</td>
<td>87</td>
</tr>
<tr>
<td>Ethanol</td>
<td>21.1 ± 3.5</td>
<td>53</td>
</tr>
<tr>
<td>Scotch whisky</td>
<td>22.2 ± 2.5</td>
<td>61</td>
</tr>
<tr>
<td>White wine</td>
<td>27.9 ± 3.4</td>
<td>102</td>
</tr>
<tr>
<td>Red burgundy wine</td>
<td>36.2 ± 3.3</td>
<td>162**</td>
</tr>
<tr>
<td>Farm-made calvados</td>
<td>21.6 ± 2.7</td>
<td>56</td>
</tr>
<tr>
<td>Commercially distilled</td>
<td>31.1 ± 3.6</td>
<td>125*</td>
</tr>
<tr>
<td>Calvados</td>
<td>36.2 ± 3.3</td>
<td>162**</td>
</tr>
<tr>
<td>Scotch whisky</td>
<td>22.2 ± 3.2</td>
<td>61</td>
</tr>
<tr>
<td>White wine</td>
<td>27.9 ± 3.4</td>
<td>102</td>
</tr>
<tr>
<td>Red burgundy wine</td>
<td>36.2 ± 3.3</td>
<td>162**</td>
</tr>
<tr>
<td>Farm-made calvados</td>
<td>21.6 ± 2.7</td>
<td>56</td>
</tr>
</tbody>
</table>

*aDetermined 3 h after a single intragastric dose of NMBzA (2.5 mg/kg; 7.4 ml/kg) in water. aqueous ethanol or alcoholic beverage (diluted to the indicated ethanol content) with tap water.

*bμmol O^-MEdG/mol dG; mean ± SD of three to six determinations in pooled DNA from five animals.

The increase in O^-MEdG levels was significantly larger than with an equivalent dose of aqueous ethanol: *P < 0.05, **P < 0.001 or ***P < 0.01.

Increasing doses of ethanol. In nasal mucosa, initial formation of O^-MEdG from NMBzA increased from 3.9 ± 1.7 μmol/mol dG in controls given tap water to 30.7 ± 1.1 μmol/mol with 4% ethanol (n = 3).

The extent of DNA methylation in oesophagus and liver of rats given NMBzA together with various alcoholic beverages was determined in a second experiment. In order to be able to detect enhancing as well as inhibitory effects on DNA methylation by congeners in the beverages beyond the modulation of NMBzA metabolism by ethanol, the beverages were diluted to a final alcohol concentration of 4% with tap water prior to administration. The results are summarized in Table II. Increases in levels of oesophageal O^-MEdG elicited by pear brandy, Japanese sake, farm-made calvados, gin, Scotch whisky, white wine or Pilsner beer (+50% to +116%) were not significantly different from that of an equivalent dose of ethanol in tap water (+87%). In contrast, significantly higher increases in the formation of O^-MEdG were observed with commercially produced apple brandy (+125%, P < 0.05) and red wine (+162%, P < 0.001). Farm-made calvados was also tested at an ethanol concentration of 20%. An increase of 200% in oesophageal DNA methylation was observed, which was significantly larger (P < 0.01) than with pure ethanol (+148%). The effects of the alcoholic beverages on levels of hepatic O^-MEdG were statistically non-significant (data not shown).

Discussion

In rats, first-pass clearance by the liver of small oral doses (<0.4 μmol/kg) of N-nitrosodimethylamine and N-nitrosodiethylamine is reduced by a concurrent dose of 10 ml/kg body wt of 5% (v/v) ethanol, leading to increased metabolism in extrahepatic tissues (18). A similar shift in bioactivation from liver to extrahepatic tissues by comparable amounts of ethanol has also been observed with nitrosamines administered at doses more than two orders of magnitude higher (19,20,27). Ethanol is a competitive inhibitor of hepatic N-nitrosodimethylamine metabolism, with a Ki, in vitro of 0.32–0.5 mM (17,18). Our results indicate that orally administered ethanol is a similarly potent modulator of nitrosamine metabolism in vivo. As shown in Figure 2, simultaneous administration of 7.4 ml/kg of 1% (v/v) ethanol, estimated to produce an initial concentration in blood of ~1.5 mM (18), was sufficient to increase oesophageal DNA methylation by a concurrent dose of 18 μmol/kg of NMBzA. It is interesting to note that though the increase in oesophageal DNA methylation was dose dependent for solutions containing 1–20% of ethanol, low doses produced a larger effect per gram of ethanol than did higher doses. Levels of O^-MEdG in liver were close to the level of quantitation, with rather large variances obscuring the effects of ethanol on NMBzA bioactivation in this tissue. A slight, albeit statistically non-significant, trend towards decreases in DNA methylation with increasing doses of ethanol was observed, however, suggesting that an inter-organ shift in NMBzA metabolism from liver to lung and oesophagus would have been detected with more sensitive methods of adduct quantitation.

The large ethanol-mediated increase in the formation of O^-MEdG from NMBzA in nasal cavity contrasts sharply with previous observations (20) of almost complete inhibition in this tissue of the bioactivation of two structurally related asymmetric nitrosamines, N-nitrosomethyl- and N-nitrosobutylamine, by a concomitant dose of ethanol. These observations provide evidence for the expression of organotropism P450 isozymes with marked differences in susceptibility to inhibition by ethanol in addition to distinct substrate specificities for carcinogenic nitrosamines. Long-term carcinogenicity assays (reviewed in ref. 28) have suggested that for certain nitrosamines, organ-selective inhibition of bioactivation by coincident ethanol plays a predominant role in the modulation of tumorigenicity and organotropism. For example, in mice, co-administration of N-nitrosodimethylamine and ethanol reduced the incidence of hepatomas but led to the induction of olfactory neuroblastomas not seen in the absence of ethanol (12). Conversely, it has been reported that ethanol given after the carcinogen had no effect on tumour induction by some nitrosamines (29–31).

Epidemiological studies have revealed that the risk for
oesophageal cancer varies markedly with the type of beverage consumed and, in Western countries, is highest for apple-based beverages (1,2). We therefore also investigated whether alcoholic beverages differ in their effects on NMBzA bioactivation. In order to be able to detect a reduction as well as an increase in oesophageal levels of O²-MedG, the beverages were adjusted to a final alcohol content of 4% with tap water. The total amounts of beverage administered were equivalent to a person of 80 kg drinking 470 ml of beer, 180 ml of red or white wine, 120 ml of sake, 50 ml of pear brandy, gin, whisky or commercial brand calvados, or 30 ml of farm-made calvados—average-sized single servings. Under these conditions, commercially distilled calvados and red wine produced significant increases in oesophageal DNA methylation beyond the enhancement observed with an equivalent amount of aqueous ethanol, suggesting that these beverages contained congeners which by themselves or in synergism with ethanol induce a significant increase in the metabolism of NMBzA. This work was supported by the Swiss National Fund.

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