

Inhibition of *N*-Nitrosodimethylamine Metabolism in Rats by Ether Anesthesia

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ABSTRACT

Short-term exposure to diethyl ether strongly inhibits the metabolism of *N*-nitrosodimethylamine (NDMA). Twenty-six 6-week-old male Fischer 344 rats were exposed to ether vapor until their righting reflex was lost (approximately 2 min). The animals were removed from the ether and NDMA was immediately administered by i.v. bolus injection at a dose of 300 $\mu\text{g}/\text{kg}$ via a cannula surgically inserted 20 h earlier. A second group of 28 rats received injections of NDMA in an identical manner but without ether exposure. In the unanesthetized animals blood levels of NDMA declined with a half-life of 11 min; by contrast essentially constant blood levels of NDMA were observed in ether-treated animals for 120 min after removal from the anesthetic. The apparent total systemic clearance for the 5-h experiment was reduced from 43 ml/min/kg without ether to 5 ml/min/kg with ether. Diethyl ether has been found previously to inhibit the metabolism of other drugs requiring oxidative metabolism but the suppression of clearance documented here appears to be unusually pronounced. It is recommended that ether's potential for altering metabolic rates be carefully considered when planning or interpreting animal experiments.

INTRODUCTION

Since the carcinogenic potential of NDMA² is not expressed unless the molecule is first metabolically activated (13) any agent that alters the efficiency of this metabolism *in vivo* might be expected to modify the carcinogenicity if administered in conjunction with the nitrosamine. Ethanol, whose interesting influence on the biological effects of NDMA has been reviewed by Swann (17), is an important example of such an agent. Recently Schmezer *et al.* (14) and Spiegelhalter *et al.* (15) have shown that ether narcosis increases urinary excretion of NDMA administered to rats by several routes indicating that this anesthetic may also be capable of inhibiting NDMA metabolism *in vivo*. Given the frequent use of diethyl ether in research with NDMA we considered it important that the effect of ether on the pharmacokinetics of the carcinogen be characterized further.

To this end we have quantified the effect of brief ether exposure on the disappearance of NDMA from the blood of Fischer rats following low dose i.v. administration. The results demonstrate that such exposure to ether is capable of suppressing almost totally the *in vivo* metabolism of NDMA and that the inhibitory effect persists long after the animals have been removed from the anesthetic.

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² The abbreviations used are: NDMA, *N*-nitrosodimethylamine; [¹⁵N₂]NDMA, [¹⁵N]nitrosodimethyl[¹⁵N]amine.

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MATERIALS AND METHODS

Chemicals. NDMA was obtained from Sigma Chemical Co., St. Louis, MO. The solution of NDMA used in all experiments was prepared by adding 52 μl of NDMA to 1000 ml of normal saline. Anhydrous ethyl ether (analytical reagent grade) was supplied by Mallinckrodt, Inc., Paris, KY. Methoxyflurane was obtained from Pitman-Moore, Inc., Washington Crossing, NJ.

Animals and Treatments. Male Fischer 344 rats (CDF F344/CrIBR) were obtained from Charles River Breeding Laboratories, Inc., Kingston, NY. The rats were supplied and maintained virus antibody-negative as determined by routinely screening sentinel animals for rat corona virus, pneumonia virus of mice, and Sendai virus. Animals were housed in Bioclean rooms (Hazleton Systems, Aberdeen, MD) maintained at a temperature of $22 \pm 1^\circ\text{C}$ (SD) and a relative humidity of $50 \pm 10\%$ with a 12-h light-dark cycle (7 a.m. to 7 p.m.) and were quarantined for 2 weeks prior to use. Animals had free access to food (Certified Rodent Chow 5002, Ralston Purina Co., St. Louis, MO) and water at all times. Rats used in these experiments weighed from 160 to 180 g and were 6 weeks of age.

To facilitate drug administration a polyethylene PE50 cannula was inserted under methoxyflurane anesthesia into the left exterior jugular vein. The cannula was threaded through s.c. tissue and skin through the back of the neck with the aid of a trocar. The cannula was flushed with a small volume of normal saline containing 10 units of heparin/ml and sealed. Twenty h later each of 28 rats received 1.00 ml of NDMA solution, 52 $\mu\text{g}/\text{ml}$, through these cannulas by bolus injection. The administered dose ranged from 286 to 310 $\mu\text{g}/\text{kg}$. Groups of animals were killed at approximately 10, 20, 40, 60, 90, 120, 180, 240, and 300 min after drug administration. Blood was collected in heparinized syringes and stored until analysis at -20°C in the presence of sodium azide (to prevent artifactual nitrosation) and morpholine (to detect artifactual nitrosation by monitoring *N*-nitrosomorpholine formation) as described previously (7). A second group of 26 rats was treated in an identical fashion except that the animals were exposed to diethyl ether vapor until the loss of their righting reflex (approximately 2 min) and were given the NDMA solution immediately upon removal from the ether.

Assay of NDMA in Blood. The concentration of NDMA in each blood sample was determined by gas chromatography-high resolution mass spectrometry using a previously described procedure (7) modified as follows. An aliquot of blood was mixed with 7.5 ng of [¹⁵N₂]NDMA in 0.3 ml of water. The mixture was diluted with 1 ml of pH 10 borate buffer (1 M) and extracted with 7 ml of dichloromethane. The organic layer was separated and concentrated to 50 μl under a stream of argon further purified by passage through concentrated sulfuric acid. A 1- μl aliquot of the concentrated solution was injected onto a 50-m x 0.34-mm flexible quartz capillary column coated with 0.2 μm of Carbowax 20 M (Chrompack, Inc.). The column was mounted in a Carlo Erba gas chromatograph and operated with oven and injector temperatures of 100°C and 128°C, respectively. The retention time of NDMA at a hydrogen carrier gas pressure of 1 kg/cm² was 180 s. The effluent from the gas chromatography column was diverted 60 s after injection directly into the chemical ionization ion source of a Kratos MS-50 high resolution mass spectrometer set to monitor, at a mass resolution of 10,000 (10% valley definition), the MH⁺ ions of NDMA at 75.0558 and [¹⁵N₂]NDMA at 77.0499 atomic mass units. The separator and ion source were at 150°C. Isobutane pressure in the ion source was 20 Pa. A small amount of isobutanol was

added to the ion source for mass scale calibration.

The m/z 75: m/z 77 ion ratio from the analysis of a blood sample was converted to a concentration of NDMA using the slope and intercept values from a weighted ($1/y$) linear least squares analysis of the ion ratio (ordinate) versus amount added (abscissa) data from the analysis in duplicate of 2-ml water samples fortified with [$^{15}\text{N}_2$]NDMA, 3.75 ng/ml, and NDMA, either 20, 5, 1.25, or 0.2 ng/ml. Various volumes of blood were analyzed in order to keep the ion ratios within the calibration range. The primary NDMA calibration solution was purchased from Thermo Electron Corp. (Waltham, MA). Precision of the analyses from consideration of the calibration curve data is as follows: interassay (relative SD of the amount found), 3.8, 9.1, 10.9, and 15.3% at 20, 5, 1.25, and 0.2 ng/ml, respectively; intraassay (relative SD of the ratio of the duplicate analyses), 6.8, 12.7, 12.3, and 19.3% at 20, 5, 1.25, and 0.2 ng/ml, respectively.

In separate experiments³ artifact formation could not be detected in any of 200 similarly prepared human plasma samples spiked with morpholine and stored at -20°C with sodium azide and morpholine for 18 mo (detection limit, 2% of the theoretical yield). One sample in the ether-treated group (at 300 min after treatment) as well as 8 of the 9 samples from the unanesthetized animals at 180 to 300 min after NDMA administration contained a nitrosamine level below the limit of quantitation (0.1 ng/ml). These values are not shown in Chart 1. In addition no NDMA was detectable in any of the 6 blank blood samples collected from animals not receiving nitrosamine with or without ether exposure.

Data Analysis. Each point from the animals not exposed to ether was normalized to a dose of 300 $\mu\text{g}/\text{kg}$, plotted and fitted to a one-compartment model with the aid of the PHARM pharmacokinetic parameter estimation program (8). Various weighting schemes were attempted including extended least squares nonlinear regression. A weight of the reciprocal of predicted value yielded the best fit as judged by examination of the residuals. The apparent blood clearance (Cl) of NDMA was calculated from the equation

$$\text{Cl} = \text{Dose}/\text{AUC}$$

where AUC = the area under the blood concentration versus time curve from time zero to infinity for the fitted data. The AUC for the ether-treated group was calculated with a linear trapezoidal rule after data of various time intervals were averaged. The AUC from time zero to the first data point was also estimated with the trapezoidal rule assuming that the concentration at time zero was 436 ng/ml (the y -intercept of the fitted data from control rats). The AUC from the last data point to time infinity was trivial and was ignored.

RESULTS

The disappearance of NDMA from blood after low (300 $\mu\text{g}/\text{kg}$) dose in rats not treated with ether was rapid (Chart 1). Although subject to significant individual variability over the time interval studied the blood concentration appeared to decline in a monoexponential manner with an initial concentration of approximately 0.4 $\mu\text{g}/\text{ml}$ and a half-life of 11 min. The calculated clearance was 43 ml/min/kg, which approached hepatic blood flow. Blood NDMA concentrations decreased to between 1 and 4% of the initial value after 1 h and to 0.2% after 2 h.

In sharp contrast the blood NDMA concentrations for animals given identical doses of NDMA following brief ether treatment while initially similar to those of the untreated group showed no significant change for the first 120 min. Three h after cessation

³ W. A. Garland, W. Kuenzig, F. Rubio, H. Holowaschenko, E. P. Norkus, J. Hess, and A. H. Conney. Effect of vitamin C and E treatment on the urinary excretion of endogenous nitrosodimethylamine and nitrosoproline in humans, submitted for publication, 1985.

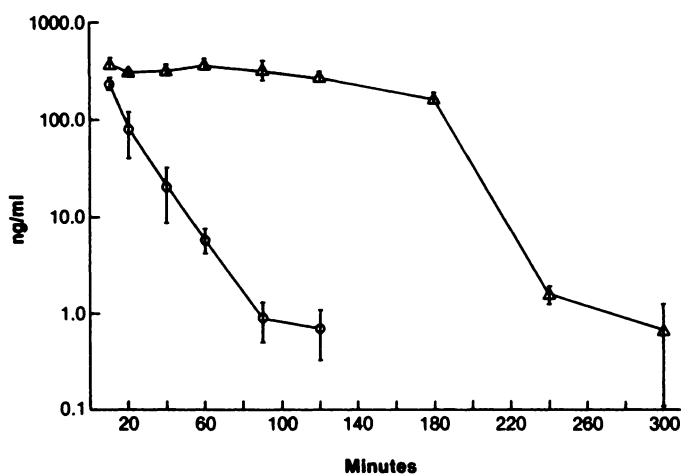


Chart 1. Blood concentrations (ng/ml) of NDMA as a function of time (min) after i.v. bolus administration to Fischer rats of the nitrosamine at a dose of 0.3 mg/kg immediately following ether anesthesia (Δ) and without ether pretreatment (\circ). Points, mean for 2-5 animals sacrificed at the same nominal time; bars, SD. Differences between ether-treated and unanesthetized groups of rats were shown to be significant at the 0.05 level using the 2-way Student's t test for all 6 time points between 10 and 120 min, inclusive.

of ether exposure and nitrosamine administration blood levels of NDMA declined at an increasing rate until they fell below the limit of detection of the analytical method at 5 h. In unanesthetized animals, on the other hand, NDMA declined to undetectable levels after 2 h. The differences were significant at the $P = 0.05$ level by the 2-way Student's t test at every time point between 10 and 120 min. The apparent clearance in the ether-treated animals over the time course of the experiment was 5 ml/min/kg.

DISCUSSION

The efficiency of the inhibitory effect of ether on NDMA metabolism in this experiment was striking. Taking the ratio of apparent clearances (43:5) as the most appropriate quantitative measure of this effect we found approximately 9-fold suppression of NDMA clearance by brief ether exposure.

If the anesthetic were continuously administered we would expect almost total inhibition of NDMA metabolism. This conclusion follows from a comparison of the initial slopes of the curves in Chart 1; NDMA levels did not detectably decline for the first 120 min in the ether-treated animals whereas NDMA was rapidly eliminated in the untreated groups. However, the initial blood concentrations obtained by extrapolating the data to time zero were similar for the 2 groups. This indicates that brief ether exposure did not dramatically alter NDMA distribution in the tissues although it is possible that both groups might have experienced an initial distribution phase. In a more comprehensive study of NDMA pharmacokinetics in unanesthetized rats we have observed⁴ that NDMA elimination is apparently biexponential with the distribution phase having a half-life of approximately 1 min. This effect cannot be seen in Chart 1 because the first time point was taken only at 10 min. A similar distribution of

⁴ B. A. Mico, J. E. Swagzdis, H. S-W. Hu, L. K. Keefer, N. F. Oldfield, and W. A. Garland. Low-dose *in vivo* pharmacokinetic and deuterium isotope effect studies of *N*-nitrosodimethylamine in rats. *Cancer Res.*, *in press*, 1985.

NDMA into the tissues of the ether-treated animals might well be observable if blood levels were examined at sufficiently early times. However, the presence of a distribution phase for control and/or ether-treated animals does not alter our conclusions about ether inhibition of NDMA's disappearance from blood.

Since most studies of NDMA carcinogenicity have involved p.o. administration of the nitrosamine it is important to consider the probable influence of brief ether exposure on the metabolism of NDMA given by this route. In the i.v. studies described above blood clearance for NDMA approached and was limited by (21) the rate of blood flow to the principal organ responsible for its metabolism (12), the liver. For NDMA administered p.o., however, the intrinsic ability of the liver enzymes to metabolize the nitrosamine would not be masked by such blood flow limitations (21). Thus the ratio of apparent clearances should be larger than that observed in the i.v. experiment because enzyme inhibition would decrease first-pass (6, 13) metabolism, increasing bioavailability in the ether-treated animals and permitting a fuller expression of the altered enzyme activity (21). In agreement with this prediction Spiegelhalter *et al.* (15) reported a 12-fold increase (relative to unanesthetized controls) in urinary NDMA excretion when the carcinogen was administered i.v. under ether anesthesia compared with a 21-fold increase for the p.o. route.

Comparison of our results with those of selected prior papers describing the effect of ether on the metabolism of other drugs (1, 3, 4, 9, 11, 18–20) indicates that the efficiency of ether suppression on NDMA metabolism may be of unprecedented magnitude. Johannessen *et al.* (11) found that antipyrine clearance was not significantly affected by exposing animals to ether for 5–10 min before drug administration, but that continuous ether anesthesia reduced the clearance by roughly 4-fold. In addition continuous ether treatment resulted in a 2.5-fold depression of aminopyrine clearance (9) relative to unanesthetized controls, an 11-fold effect on phenytoin metabolism (18) and a 1.5-fold inhibition of hexobarbital elimination (20). After only 2 min of ether exposure we observed nearly complete inhibition of NDMA disappearance during the first 2 h and an apparent 9-fold suppression of clearance over the 5 h of the experiment.

One possible reason for the larger effect in our experiments is that the enzymes involved in NDMA metabolism may be different from those that metabolize the other drugs. Another perhaps more probable explanation is suggested by examination of the doses of test substrate used in these studies. We administered NDMA at doses of 4 $\mu\text{mol/kg}$ whereas doses in the other studies were 80 $\mu\text{mol/kg}$ for antipyrine, 130 $\mu\text{mol/kg}$ for aminopyrine, 40 $\mu\text{mol/kg}$ for phenytoin, and 190 $\mu\text{mol/kg}$ for sodium hexobarbital. If inhibition of drug metabolism by ether is a competitive process as has been shown for demethylation of *p*-nitroanisole (10) then the degree of inhibition is a function of inhibitor concentration, the inhibition constant, and the concentration of test substrate. Since we used low doses of test substrate it is possible that the observed inhibitory effect was greater than it might have been if NDMA blood concentrations had been higher. A corollary of this hypothesis is that low dose NDMA metabolism may be a sensitive experimental model for further studies of inhibition of oxidative metabolism.

As to the mechanism of ether inhibition of NDMA metabolism

the explanation that the effect is a direct consequence of anesthesia can probably be excluded because the quantity of ether administered was minimal and the inhibition of metabolism persisted long after the animals had regained their normal behavior. Also anesthesia with a combination of dehydrobenzperidol and fentanyl decreases urinary excretion of NDMA (14). Nevertheless the possibility that other anesthetics (especially those which are chemically related to ether) may also act as metabolic inhibitors (10) should be kept in mind until data on their potential for such activity become more widely available.

Since ether is known to undergo oxidative metabolism to acetaldehyde and ethanol (2, 5, 16) it is conceivable that the observed inhibition is due to the known (17) effects of ethanol on NDMA metabolism. Although this possibility cannot be completely excluded it appears unlikely given the short duration of exposure and the lack of extensive metabolism of ether (2).

In conclusion we strongly recommend that those contemplating the collection or interpretation of data on xenobiotic metabolism be particularly mindful of the potential for complications which may be encountered when exposure to diethyl ether is included in the protocol. This caveat may assume particular importance in studying nitrosamine pharmacokinetics, since inhibition was much more pronounced for NDMA metabolism than was observed in previous investigations with other drugs.

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